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1 Supramolecular Peptide Nanofiber Morphology

2 Affects Mechanotransduction of Stem Cells

3 Elif Arslan¹, Meryem Hatip Koc¹, Ozge Uysal^{1, #}, Begum Dikecoglu¹, Ahmet E. Topal¹,
4 Ruslan Garifullin^{1, 2}, Alper D. Ozkan¹, Aykutlu Dana¹, Daniel Hermida-Merino³, Valeria
5 Castelletto⁴, Charlotte Edwards-Gayle⁴, Sefer Baday⁵, Ian Hamley⁴, Ayse B. Tekinay^{1, #}
6 *, Mustafa O. Guler^{6, *}

7 *¹Institute of Materials Science and Nanotechnology and National Nanotechnology*
8 *Research Center (UNAM), [#]Neuroscience Graduate Program, Bilkent University, Ankara*
9 *06800, Turkey*

10 *²Institute of Fundamental Medicine and Biology, Kazan Federal University, 420021*
11 *Kazan, Russian Federation*

12 *³ESRF – The European Synchrotron, 38000 Grenoble, France*

13 *⁴Department of Chemistry, University of Reading, Whiteknights, Reading RG6 6AD, U.K.*

14 *⁵Applied Informatics Department, Informatics Institute, Istanbul Technical University,*
15 *Istanbul, 34469, Turkey*

16 *⁶Institute for Molecular Engineering, University of Chicago, Chicago, IL 60637, USA*

17 *Corresponding authors' e-mail addresses: mguler@uchicago.edu (M.O.G.) and
18 atekinay@bilkent.edu.tr (A.B.T.)

19

20 **KEYWORDS**

21 Supramolecular peptide nanofibers, mechanotransduction, stem cells, chirality,
22 morphology

1 **ABSTRACT**

2 Chirality and morphology are essential factors for protein function and interactions with
3 other biomacromolecules. Extracellular matrix (ECM) proteins are also similar to other
4 proteins in this sense; however, the complexity of the natural ECM makes it difficult to
5 study these factors at the cellular level. The synthetic peptide nanomaterials harbor great
6 promise in mimicking specific ECM molecules as model systems. In this work, we
7 demonstrate that mechanosensory responses of stem cells are directly regulated by the
8 chirality and morphology of ECM-mimetic peptide nanofibers with strictly controlled
9 characteristics. Structural signals presented on L-amino acid containing cylindrical
10 nanofibers (L-VV) favored the formation of integrin β 1-based focal adhesion complexes,
11 which increased the osteogenic potential of stem cells through the activation of nuclear
12 YAP. On the other hand, twisted ribbon-like nanofibers (L-FF and D-FF) guided the cells
13 into round shapes and decreased the formation of focal adhesion complexes resulting in
14 the confinement of YAP proteins in the cytosol and a corresponding decrease in
15 osteogenic potential. Interestingly, the D-form of twisted-ribbon like nanofibers (D-FF)
16 increased the chondrogenic potential of stem cells more than their L-form (L-FF). Our
17 results provide new insights into the importance and relevance of morphology and
18 chirality of nanomaterials in their interactions with cells, and reveal that precise control
19 over the chemical and physical properties of nanostructures can affect stem cell fate even
20 without the incorporation of specific epitopes.

21 **INTRODUCTION**

22 In their native microenvironment, cells respond to a broad range of extracellular matrix
23 (ECM) signals by modulating their mechanical properties through cytoskeletal
24 remodeling.¹ The ECM provides a physical scaffold that is integral for the transduction of
25 biochemical and biomechanical signals necessary for proper functioning of cells and
26 tissues. Structural and chemical features of ECM elements are essential for their ability to
27 elicit specific cellular responses, and synergistic interactions between these signals are
28 also crucial for the regulation of cellular behavior. For example, integrin-binding motifs
29 such as GFOGER occur on collagen and only exhibit bioactivity when presented within a
30 triple helix structure.² Thus, the macromolecular structure can substantially alter the

1 context of even well-established biochemical signals by modulating their interactions
2 with cellular receptors.³

3 Stem cells adapt environmental signals as biochemical information through contractile
4 forces acting on fibronectin fibrils.⁴ In addition, fibrillar proteins in the ECM exhibit
5 certain structural fingerprints that impart them with their fundamental functions. For
6 example, different collagen types in various supramolecular structures; including
7 geometric networks, membrane-spanning fibrils, and beaded-filaments, contribute to a
8 diverse range of functions such as providing tensile strength and enabling resistance to
9 plastic deformation and rupture.⁵ In addition, collagen fibrils exhibit a periodicity known
10 as the D-band, which determines the stiffness of the microenvironment. Alterations in
11 this periodicity are known to result in disease due to disorders in the shape and
12 mechanical strength of the fibrillar structure.⁶

13 Supramolecular structures of the ECM fibrillar proteins are tissue-specific and their
14 mechanical properties are optimized to react to the specific range of external and internal
15 forces that is routinely experienced by each tissue. Through these proteins, externally
16 applied mechanical forces function at the molecular level by regulating focal adhesion
17 (FA) point size, shape, and composition in cells.⁷ In addition to supramolecular
18 structures, chirality is also a characteristic of many biomacromolecular interactions that
19 govern cellular behaviors.⁸⁻¹⁰ In fact, a wide range of biomacromolecules function
20 precisely due to specific enantiomeric interactions that fail to occur if both partners do
21 not share a chiral configuration.¹¹ If any particular component was to be replaced by an
22 unsuitable enantiomeric counterpart, the function of the whole system would be lost due
23 to the ensuing destabilization effect that distorts the secondary structure of proteins and
24 other biomacromolecules.¹²

25 Inspired by the features of tissues and their ECM, a broad range of functional therapeutic
26 biomaterials have been developed by emulating the physical, chemical and biological
27 properties of native tissue microenvironments.¹³⁻¹⁵ Among these biomaterials, peptide
28 nanofibers have great potential in mimicking natural ECM by incorporating specific
29 signal sequences.^{16, 17} While the biological activity of peptide nanofibers is typically
30 based on the cellular recognition of their functional epitope sequences, their physical and
31 chemical properties are also essential for mediating cell-biomaterials interactions.⁷

1 Hence, self-assembled supramolecular peptide amphiphile nanofibers provide a useful
2 toolkit for the investigation of cell-ECM interactions by exhibiting a diversity of
3 mechanical properties despite their simple, well-defined and highly bioactive
4 structures.¹⁸⁻²⁰ Self-assembly can be triggered through different external and internal
5 forces while providing an extremely large morphological diversity and various physical
6 and chemical features as a result.^{21,22} Several studies have demonstrated that nanofibers
7 with distinct morphology and chirality are able to selectively interact with cells to elicit
8 specific cellular responses, but these studies only focus on one of these features in
9 isolation from the others.^{6, 8, 9, 23} However, emulating the complexity inherent to native
10 tissues requires sophisticated biomaterials design integrating distinctive features to
11 precisely regulate the distribution of cells, macromolecules and structural elements at
12 various scales and dimensions²⁴.

13 In this work, we demonstrated peptide nanofibers with strictly controlled morphology and
14 chirality to study the effects of these features on the cellular fate at the molecular and
15 cellular levels. We monitored the control of cellular responses, including the
16 differentiation of stem cells, by tuning the physical and chemical cues provided by the
17 peptide nanofiber materials. Twisted ribbon-like nanofibers (L-FF and D-FF) reduced
18 cellular spreading as well as ERK/MAPK pathway activity, resulting in genetic
19 regulation through the repression of nuclear YAP (Yes-associated protein) activity, which
20 reduced the osteogenic differentiation of stem cells while favoring chondrogenesis by
21 altering cell shape through matrix-induced cellular rounding. In contrast, the L- amino
22 acid containing cylindrical nanofibers (L-VV) produced a greater mechanical feedback
23 and enhanced cellular spreading through increased interaction with the integrin β 1
24 receptor. Stem cells on L-VV scaffolds activated the ERK/MAPK pathway, which
25 resulted in YAP/TAZ activation and nuclear YAP localization, which further increased
26 the osteogenic differentiation of stem cells. These results demonstrate the importance of
27 morphology and chirality on effect of ECM-mimetic nanofibers on stem and somatic cell
28 behavior, and show that the behavior of stem cells could be tuned even without the
29 incorporation of biologically relevant epitopes.

30 **Experimental Section**

Materials

9-Fluorenylmethoxycarbonyl (Fmoc) and tert-butoxycarbonyl (Boc) protected amino acids, [4-[α -(2',4'-dimethoxyphenyl) Fmoc aminomethyl]enoxy] acetamidonorleucyl-MBHA resin (Rink amide MBHA resin), Fmoc-Glu(OtBu)-Wang resin and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from NovaBiochem and ABCR. Cover glasses and tissue culture plates (24-well) were purchased from Deckglaser and BD. All other chemicals and materials used were analytical grade and obtained from Invitrogen, Fisher, Merck, Alfa Aesar and Sigma-Aldrich. Chondrogenic differentiation media were purchased from Sigma. Live/Dead Assay (L3224), Alamar Blue and other cell culture materials were purchased from Invitrogen. Western blotting, flow cytometry, and immunocytochemistry antibodies were purchased from Abcam, Millipore, or Santa Cruz Biotechnologies: anti-vinculin antibody, (Abcam, ab18058), anti-FAK antibody (Abcam, ab72140), mouse monoclonal IgG2a (Abcam, ab170191), goat anti-mouse IgG H&L (Alexa Fluor® 488) (Abcam, ab150113), anti-Integrin α 2 antibody [EPR17338] - C-terminal (Abcam, ab181548), anti-FAK antibody [63D5] (Abcam, ab72140), anti-phospho-FAK (Tyr397) antibody clone EP2160Y (Millipore, 04-974), anti-ERK1 + ERK2 antibody [IL-13] (Abcam, ab130004), anti-ERK1 (pT202/pY204) + ERK2 (pT185/pY187) antibody [MAPK-YT] (Abcam, ab50011), anti-MEK1 + MEK2 antibody (ab178876) or anti-phospho-MEK1 (Ser218/222)/MEK2 (Ser222/226) antibody (Millipore, 05-747), Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (Abcam, ab150113), YAP antibody (H-9) (SCBT, sc-271134).

Synthesis and Characterization of Peptide Amphiphile Molecules

All peptides were synthesized by using Fmoc solid phase peptide synthesis. All peptides including lauric acid were constructed on Fmoc-Rink Amide MBHA resin. Amino acid coupling reactions were performed with 2 equivalents of Fmoc-protected amino acid, 1.95 equivalents of HBTU and 3 equivalents of DIEA for 2 h. The Fmoc protecting group removal was performed with 20% piperidine/DMF solution for 25 min. Cleavage of the peptides from the resin was carried out with a mixture of trifluoroacetic acid (TFA) : triisopropylsilane (TIS) : H₂O at a ratio of 95 : 2.5 : 2.5 for 2 h. Excess TFA was removed by rotary evaporation. The remaining peptide was triturated with ice-cold diethyl ether and the resulting white precipitate was freeze-dried. All peptides were purified by

Preparative Liquid Chromatography (Prep-HPLC) and positively charged peptides were treated with 1 mM HCl.

Before each characterization, 1% (w/v) EE-PA solutions and KK-PA solutions were dissolved in water separately. Four samples; L-VV, D-VV, L-FF, D-FF, which are listed in Table 1, were prepared by mixing positively and negatively charged peptide solution pairs at 1:1 volume ratio (Table 1). These neutralized samples were incubated overnight and all characterizations were done at the physiological pH.

Table 1. List of positively and negatively charged peptide amphiphiles

Networks	Positive PA	Negative PA
L-VV	Lauryl-VVAGKK-Am	Lauryl-VVAGEE-Am
D-VV	Lauryl-vvaGkk-Am	Lauryl-vvaGee-Am
L-FF	Lauryl-FFAGKK-Am	Lauryl-FFAGEE-Am
D-FF	Lauryl-ffaGkk-Am	Lauryl-ffaGee-Am

Liquid Chromatography and Mass Spectrometry (LC-MS)

Agilent Technologies 6530 Accurate-Mass Q-TOF-MS equipped with HPLC using Zorbax SB-C8 column was used for LC-MS analysis. LC-MS sample was prepared in 0.5 mg/mL concentration. Mobile phase solutions were water (0.1% formic acid) and acetonitrile (ACN) (0.1% formic acid). LC-MS was run for 30 min for each sample and it started with 2% ACN and 98% H₂O for 5 min. Then, gradient of ACN reached 100% in 20 min. Finally, its concentration was dropped to 2% and it was kept running for 5 min. Solvent flow was 0.65 mL/min and 5 μ L sample was injected.

Circular Dichroism (CD)

A Jasco J-815 CD spectrophotometer was used for CD analysis. 1% (w/v) positively and negatively charged peptide solution mixtures were diluted first to 2 mM, then to 0.25 mM concentration, gradually. This prevented the disintegration of the co-assembled network through dilution. 0.25 mM solutions were used for the CD measurement in 1 mm quartz cell. Peptide solution was measured from 300 nm to 190 nm with 0.1 data pitch, 100

1 nm/min scanning speed, 1 nm bandwidth and 4 s D.I.T. Average of three measurements
2 were used, and sensitivity was selected as standard.

3 Transmission Electron Microscopy (TEM)

4 Imaging of the peptide nanostructures was achieved by TEM (FEI, Tecnai G2 F30) at
5 100 kV. For peptide nanofiber staining, uranyl acetate solution in water (2 wt %) was
6 used. Each of four 1% (w/v) peptide stock solutions were gradually diluted to 2 mM
7 solution, then to 50 μ M TEM sample solution. These diluted samples were placed on a
8 Lacey carbon coated copper grid. 10 μ L of diluted sample solution was dropped on a grid
9 and kept there for 8 min. The excess was removed by pipette. Then, 20 μ L of 2 wt %
10 uranyl acetate solution was put on a parafilm sheet. The grid was placed on the top of the
11 drop with its upper side down and kept there for 5 min. Stained grids were dried in a
12 fume hood at room temperature overnight.

13 Small-Angle X-ray Scattering (SAXS)

14 Experiments on single component peptide solutions were performed on beamline B21 at
15 Diamond Light Source, Harwell, UK. Solutions (1 wt % or 0.5 wt % for more viscous
16 samples) were loaded into the 96 well plate of an EMBL BioSAXS robot. Aliquots of
17 solutions (25 μ L) were then injected via an automated sample exchanger at a slow and
18 very reproducible flux into a quartz capillary (1.8 mm internal diameter) in the X-ray
19 beam. For D-VVEE, the 1 wt % sample was too viscous to flow into the capillary,
20 however, dilution to 0.5 wt % enabled sample delivery into the beam. The quartz
21 capillary was enclosed in a vacuum chamber, in order to avoid parasitic scattering. After
22 the sample was injected in the capillary and reached the X-ray beam, the flow was
23 stopped during the SAXS data acquisition. SAXS frames were collected with duration of
24 20 s or 100 s). B21 operated with a fixed camera length (4.01 m) and fixed energy (12.4
25 keV). The images were captured using a Pilatus 2M detector. Data processing
26 (background subtraction, radial averaging) was performed using the dedicated beamline
27 software Scatter.

28 SAXS data were modeled using the software SASfit²⁵ with model “Bilayer Gauss”. This
29 model describes tape-like structures represented as bilayers with electron dense cores and
30 lower electron density surfaces. The model, used in several previous papers^{26, 27} was that
31 of Pabst et al.²⁸ In some cases (data for L-FFEE and D-FFKK) an alternative nanotube

1 form factor provided a better fit to the data at low q where a maximum is observed in the
2 intensity, corresponding to helically wrapped nanotapes. The fitting was done using
3 SASfit²⁵. A flat background was added in the model for all data.

4 Simultaneous SAXS/WAXS experiments on gel-forming mixtures were performed on
5 beamline BM26B at the ESRF. Samples were placed in DSC pans modified with mica
6 windows to enable transmission of the X-ray beam. The sample to SAXS detector
7 distance was 3.16 m using a wavelength of 1.033 Å. A Dectris-Pilatus 1 M detector with
8 a resolution of 981×1043 pixels and a pixel size of 172×172 μm was used to acquire
9 the 2D SAXS scattering patterns. Standard corrections for sample absorption and
10 background subtraction were performed. The data were normalized to the intensity of the
11 incident beam (in order to correct for primary beam intensity fluctuations) and were
12 corrected for absorption and background scattering. Diffraction from silver behenate was
13 used to calibrate the wavevector scale of the scattering curve.

14 Molecular Dynamics Simulations

15 All-atom explicit solvent simulation systems were prepared for four PA nanofibers. Each
16 PA nanofiber system was constructed using 18 layers that were composed of 12 PAs. The
17 starting configuration for each PA nanofiber was done based on previous simulations,
18 such that 19 layer with 12 PA in each layer configuration gave rise to the most stable
19 configuration for PAs having similar length compared with PAs given in this work²⁹. In
20 order to maintain 1:1 stoichiometry between glutamate (GLU-G) and lysine (LYS-K)
21 having peptides, adjacent layers were constructed with either GLU only or LYS only
22 peptides. To establish ionic interactions between the main simulation box and periodic
23 images, 18 layers were chosen instead of 19 layers. Each layer was built by placing 12
24 PAs with 30° angle separation. Adjacent layers were put together with 5 Å distance away
25 and 15° angle rotation (Figure S8). The PA nanofibers were solvated with TIP3 water
26 molecules and, Na⁺ and Cl⁻ ions were added to reach 0.15 M salt concentration. Resulting
27 simulation system boxes contained around 120,000 atoms.

28 MD simulations for the PA nanofibers were performed using NAMD program (version
29 2.9) with CHARMM force field^{30, 31}. Prior to production simulations, simulation systems
30 were minimized with 1000 minimization steps. 100 ns production simulations were
31 carried out for each PA nanofiber system at 1 atm pressure and 310 K temperature.

Electrostatic interactions were calculated using the particle-mesh Ewald method with a grid spacing³². The cutoff for van der Waals interactions was taken as 12 Å with a switching function after 10 Å. Simulation trajectories were integrated with a time step of 2 fs, with all interactions calculated at every time step. Atomic coordinates were collected every 10 ps. The analyses were applied to the last 20 ns of each trajectory. Hydrogen bonds and radial distribution functions (RDF) were calculated using CPPTRAJ program³³. Non-bonded interaction energy calculations on simulation trajectories were carried out using VMD program³⁴.

Nanomechanical Characterization of Peptide Nanofibers by Atomic Force Microscopy (AFM)

For AFM measurements, peptide nanofibers were prepared as 5 µM in same method with TEM imaging protocol, and were dropped onto a freshly cleaved mica surface. Silicon nitride (Budget Sensors) AFM probes were used for contact mode imaging of the self-assembled peptide nanofibers in liquid. For performing of force mapping in contact-mode, Asylum Research MFP-3D AFM was used. Force maps were taken at a resolution of 32 x 32 from a 1-5 µM area. A trigger point of 0.5 V was applied for curve measurements. Vertical deflection correction was performed before starting force map measurements. A total of more than 800 force curves were analyzed per peptide nanofiber group for AFM analysis. The Hertz model was applied to calculate elastic moduli from approach curves. The Poisson ratio of the nanofibers was assumed to be 0.33.

Oscillatory Rheology Analysis

Oscillatory rheology measurements were performed with an Anton Paar Physica MCR301 system. A 25 mm parallel plate with a gap distance of 0.5 mm was used at 25 °C for all measurements. The total gel volume was adjusted as 250 µL. PA solutions were freshly prepared as 10 mM and sonicated for 30 min. Gels were prepared using the combinations seen in Table S1. The negatively charged PAs were first loaded at the center of the stage and mixed with the positively charged PAs. The upper plate was adjusted to the 0.5 mm position and the gel was incubated in this position for 15 min prior to measurement. For strain sweep measurements, angular frequency was kept constant at 10 rad/s, and strain was increased between 0.1 and 100%. Storage and loss

moduli were recorded at each strain value. All rheology measurements were with three replicates.

Scanning Electron Microscopy (SEM) Analysis

For SEM imaging, 1 % (w/v) bulk peptide nanofiber gels were prepared on silicon wafers. Samples were dehydrated in graded ethanol solutions, starting with 20% ethanol and proceeding to absolute ethanol for 10 min at each step. Samples were dried with a Tourismis Autosamdri-815B critical point drier, coated with 10 nm Au/Pd and imaged with a FEI Quanta 200 FEG SEM.

In vitro cell culture experiments

Peptide amphiphile nanofiber scaffolds were prepared through the protocols used for chemical characterization experiments. Briefly, PA solutions were prepared in ddH₂O at a concentration of 2 mM and sterilized under UV for 1 h. Then, the corresponding well plates (96-well plates or 24-well plates, depending on the experiment) were coated with PA combinations (Table S1). Peptide coated plates were sealed and incubated at room temperature overnight for hydrogel consolidation before use. rMSCs (rat mesenchymal stem cells, Invitrogen S1601-100, at passage 6-8), HUVECs (HUVECs were kindly provided by Yeditepe University, Istanbul, Turkey) and primary human fibroblasts (hFib) (hFibs were kindly provided by Middle East Technical University, Ankara, Turkey) were used in in vitro cell culture experiments. All cells were initially cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (maintenance medium, MT) in tissue culture plates at standard culture conditions (at 37 °C under 5% CO₂). For differentiation analyses, after 1 day of incubation for cell attachment, the medium was replaced with fresh MT, chondrogenic differentiation medium (Gibco) or osteogenic medium. MT supplemented with 10 mM β -glycerophosphate, 0.2 mM ascorbic acid and 100 nM dexamethasone was used as an osteogenic medium for the osteogenic differentiation of rMSCs.

Viability analysis

Cell cultures were prepared in 96 well plates in MT medium, and cellular viability was analyzed by Alamar Blue (Invitrogen) and Live/Dead (Life Technologies) assays. 5000 rMSCs, HUVECs, or hFibs were seeded onto peptide coatings and their viability was measured at 24 h, 48 h and 72 h by measuring the fluorescence and absorbance of the

1 reagent by spectrophotometry for the Alamar Blue assay, and imaging the cells by
2 fluorescence microscopy for the Live/Dead Assay.

3 Adhesion analysis

4 Cell cultures were prepared in 96-well plates (2000 cells/well, 3 replicas for each peptide
5 nanofiber group) in MT medium and cellular adhesion was analyzed by using adhesion
6 medium, which is serum free MT medium including 50 µg/mL cyclohexamide and 4
7 mg/mL BSA. Before seeding, cells were incubated in adhesion medium for 1 h and
8 subsequently seeded onto peptide coatings in adhesion medium. After 1 h and 5 h, wells
9 were washed with PBS and adherent cells were stained with Calcein Am for 20 min. The
10 number of adherent cells were counted using Image J from fluorescence microscopy
11 images.

12 Proliferation analysis

13 Cell cultures were prepared in 96-well plates in MT medium and cellular proliferation
14 was analyzed by a colorimetric ELISA-based BrdU assay (Cell Proliferation ELISA,
15 BrdU; Roche) according to the manufacturer's protocol. Briefly, 5000 cells/well were
16 seeded onto coatings, and after 1, 3 and 5 days, BrdU assay was performed by incubating
17 cells with BrdU labeling reagent, fixing them, and staining them with anti-BrdU-POD. A
18 manufacturer-provided colorimetric substrate was added to the wells for the development
19 of antibody staining, and absorbance values were analyzed by a spectrophotometer.

20 Spreading analysis

21 Cell cultures were prepared in 24-well plates on glass cover slides in MT medium and
22 cellular spreading was analyzed by staining cells with Phalloidine/TO-PRO-3
23 (Invitrogen). Images were taken by fluorescence microscopy, and analyzed by Image J
24 software.

25 Gene expression analysis

26 Cell cultures (rMSC) were prepared in 24-well plates in MT or chondrogenic
27 differentiation medium. Gene expression analyses were performed by amplifying markers
28 for chondrogenesis (Sox-9) and osteogenesis (Runx-2) by quantitative RT-PCR (qRT-
29 PCR). Total RNAs of rMSCs on peptide coatings were isolated using TRIzol (Invitrogen)
30 according to the manufacturer's instructions. Yields and purities of extracted RNA were
31 assessed by Nanodrop 2000 (Thermo Scientific). Primer sequences were designed using

1 Primer 3 software (Table S2). cDNA synthesis from RNA and qRT-PCR were performed
2 using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen)
3 according to the manufacturer's protocol. Reaction conditions were briefly as follows: 55
4 °C for 5 min, 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 40 °C for 1
5 min, followed by a melting curve to confirm product specificity. Reaction efficiencies
6 were evaluated for each primer set through standard curves using 5-fold serial dilutions
7 of total RNA. For the analysis of expression, primary gene expression data were
8 normalized by the expression level of GAPDH. A comparative Ct method was used to
9 analyze the results.

10 Protein expression analyses by flow cytometry

11 Flow cytometry was performed to quantify the expression of Vinculin and FAK proteins,
12 which are important regulators of focal adhesion and cellular mechanotransduction. Prior
13 to flow cytometry analysis, the cells were disintegrated from peptide coatings by
14 collagenase/trypsin treatment. The supernatant was then collected and centrifuged at
15 2500 rpm for 5 min. The cell pellet was washed twice with PBS, resuspended, and fixed
16 with 4% paraformaldehyde for 15 min at room temperature. The solution was then
17 centrifuged at 2500 rpm for 5 min, and the cell pellet was resuspended and permeabilized
18 in 0.2% Tween-20/PBS for 15 min at room temperature. A primary antibody solution
19 (either Vinculin [anti-Vinculin antibody, (ab18058)] or FAK [anti-FAK antibody
20 (ab72140)]) or their corresponding isotype (mouse monoclonal IgG2a (ab170191)) was
21 prepared in 3% BSA/PBS solution at concentrations recommended by the manufacturer
22 (Abcam) and used to stain the cells for 1 h. After primary antibody staining, cells were
23 washed with permeabilizing solution, pelleted by centrifugation and stained with
24 secondary antibodies [goat anti-mouse IgG H&L (Alexa Fluor® 488) (ab150113)] for 1
25 h. After washing twice with permeabilizing agent, the cells were again pelleted by
26 centrifugation and resuspended in PBS prior to cytometry analysis. A BD Accuri™ C6
27 flow cytometer system was used for cytometry and at least 20,000 events were analyzed
28 for the measurements. For data analysis, BD Accuri™ C6 software was used according to
29 the manufacturer's instructions.

30 Immunoblotting analysis of protein expression by western blotting

Western blotting analyses were performed to detect the expression levels of key proteins of mechanotransduction and the associated molecular pathways. Cells were isolated from their peptide coatings by RIPA buffer (including protease and phosphatase inhibitor cocktail), and the protein containing supernatant was removed and stored at -80 °C. Protein concentrations were determined using BCA Protein Assay Kit. Equal amounts of proteins per lane were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) or nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBS-T at room temperature for 2 h and then incubated with anti-Integrin $\alpha 2$ antibody [EPR17338] - C-terminal (Abcam, ab181548), anti-FAK antibody [63D5] (Abcam, ab72140), anti-phospho-FAK (Tyr397) antibody clone EP2160Y (Millipore, 04-974), anti-ERK1 + ERK2 antibody [IL-13] (Abcam, ab130004), anti-ERK1 (pT202/pY204) + ERK2 (pT185/pY187) antibody [MAPK-YT] (Abcam, ab50011), anti-MEK1 + MEK2 antibody (ab178876) or anti-phospho-MEK1 (Ser218/222)/MEK2 (Ser222/226) antibody (Millipore, 05-747) overnight at 4 °C. After washing in TBS-T, the blots were incubated with the corresponding horseradish-coupled secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG). The bands were visualized using Clarity™ Western ECL blotting substrate. GAPDH (Millipore) was used as the internal control and treated with the same protocol. Protein amounts in each sample were quantified using ImageJ software.

Immunocytochemical analyses of protein detection by confocal microscopy

The rMSCs on peptide coatings were fixed in 4% paraformaldehyde/PBS for 10 min and permeabilized in 0.1% Triton X-100 for 15 min. For blocking, samples were incubated with 3% (w/v) bovine serum albumin/PBS for 30 min and treated with either YAP antibody (H-9) (SCBT, sc-271134) or anti-Vinculin antibody [SPM227] (ab18058) overnight at 4 °C. Cells were then washed with PBS and incubated for 1 h at room temperature with goat anti-mouse IgG H&L (Alexa Fluor® 488). All samples were counterstained with 1 μ M TO-PRO-3 (Invitrogen) in PBS for 20 min at room temperature and mounted with Prolong Gold Antifade Reagent (Invitrogen). Negative controls were obtained by omitting the primary antibody and incubating with 3% normal goat serum/PBS. Samples were imaged by confocal microscopy (Zeiss LSM510) and analyzed

by Image J program for cell counting to determine the cell amount with nuclear YAP localization.

Collagen adsorption analyses by ELISA

For the determination collagen I adsorption on peptide nanofibers, indirect-ELISA was performed. ELISA plates were coated with peptide nanofibers. On the next day, plates were washed by washing buffer, dried by tapping, blocked with assay buffer (Life Technologies, DS98200) (2 h) and Collagen I (Millipore, 08-115) was added onto peptide coatings for overnight at 4 °C. Next day, plates were washed by washing buffer, dried by tapping, then they were incubated with anti-collagen I (1:500) (ab6308) primary antibody for overnight at 4 °C. After that, plates were washed 5 times with washing buffer and dried by tapping between each consecutive step. The HRP-conjugated anti-IgG antibody was used as a secondary antibody and incubated for 2 h. The TMB (3,3',5,5'-tetramethylbenzidine) substrate was added at the last step and the reaction was stopped after 15 min with stop solution (1.8 N H₂SO₄). Color formation was measured by using a microplate reader (Spectramax M5, Microplate reader) as absorbance at 450 nm wavelength (reference absorbance measured at 650 nm and subtracted from absorbance at 450 nm). All treatments were performed with at least four replicates and peptide nanofiber coatings without collagen I incubation were used as blank.

Statistical analysis

All data are presented as mean \pm Sem (standard error of mean). All experiments were performed in at least three replicas. The significance of differences between groups was determined with either one-way or two-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. Differences were considered significant at *p<0.05, except where noted.

RESULTS

Design, preparation and characterization of self-assembled PA molecules

Here we designed four different peptide nanofibers with distinct morphology and chirality, and evaluated their interactions with cells at the molecular level. Each nanofiber consisted of two oppositely charged peptide amphiphile (PA) molecules. In order to obtain four peptide nanofiber types (cylindrical L-form, cylindrical D-form, ribbon-like L-form, ribbon-like D-form), eight peptide amphiphile molecules, each consisting of a

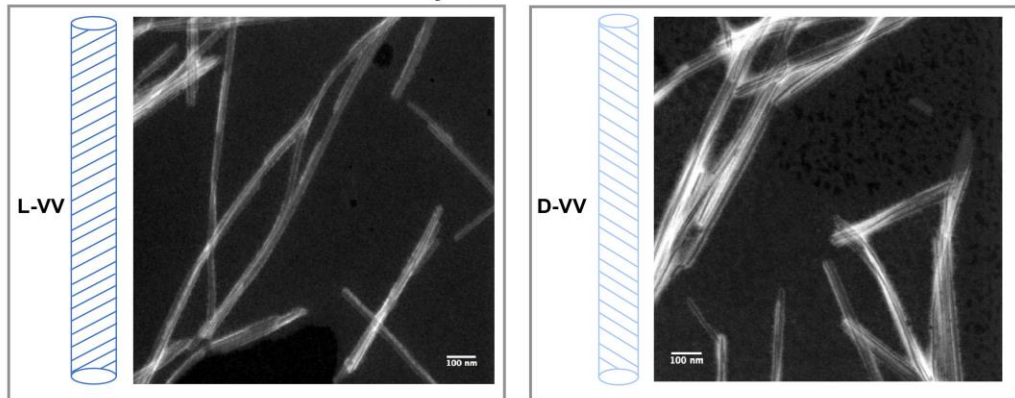
hydrophobic region, a β -sheet forming region, and a hydrophilic (or charged) region were designed and synthesized in two different chiral forms (Table 1). The hydrophobic region contained a lauric acid alkyl tail that is long enough to trigger specific nanofiber organization in water.¹³ Either two glutamic acid or two lysine residues were used for the hydrophilic region. Four PA molecules were synthesized with a charge of +2, and another four of them with a charge of -2 at the physiological conditions (Table 1). Two oppositely charged PA molecules co-assembled into high-aspect-ratio nanostructures through electrostatic interactions between positively and negatively charged building blocks to form each nanofiber network.³⁵ In addition, hydrophobic and hydrophilic regions of each PA molecule were linked together with a hydrogen bonding peptide region, which was essential for modulating self-assembly behavior to form cylindrical or ribbon-like structures. The valine-valine sequence caused the highest propensity for forming β -sheets for cylindrical nanofiber formation.^{36, 37} The phenylalanine-phenylalanine sequence produced a twisted ribbon morphology through π - π stacking, which is the major promoting factor of the twisted morphology in the co-assembled network for ribbon-like nanostructure formation.³⁸ In addition, for achieving chiral differences in networks, all peptides were synthesized in both L and D enantiomeric forms.³⁹ Successful synthesis and purification of all eight PAs were confirmed by prep-HPLC and LC-MS (Figures S1 and S2). Morphology of the PA nanostructures was investigated by transmission electron microscopy (TEM) (Figure 1A). While L-VV and D-VV produced cylindrical nanofibers, twisted ribbon morphologies were observed for L-FF and D-FF. Uniform width and pitch sizes were obtained for L-FF and D-FF. The L-FF ribbons had 21.21 (\pm 3.7) nm average diameters and 55.70 (\pm 8.6) nm average pitch size, while D-FF formed ribbons that had an average diameter of 18.21 (\pm 2.7) nm and pitch size of 48.21 (\pm 7.3) nm (Figures S3 and S4). SAXS analysis showed that the form factor features of the eight PA solutions were characteristic of nanotape structures. SAXS data with model form factor fits are shown in Figure S6. The fits exhibited layer thicknesses of 38 ± 2 Å for L-VV and D-VV samples and 29 ± 1.5 Å for L-FF and D-FF samples. This is consistent with completely interdigitated bilayer structures (*i.e.* fully overlapped lipid chains in the bilayer interior), since the length of a PA molecule packed in a parallel β -sheet is estimated to be 37 Å.

1 For all four gels, SAXS showed very similar form factors (Figure S7) that exhibited
2 features of twisted ribbons.

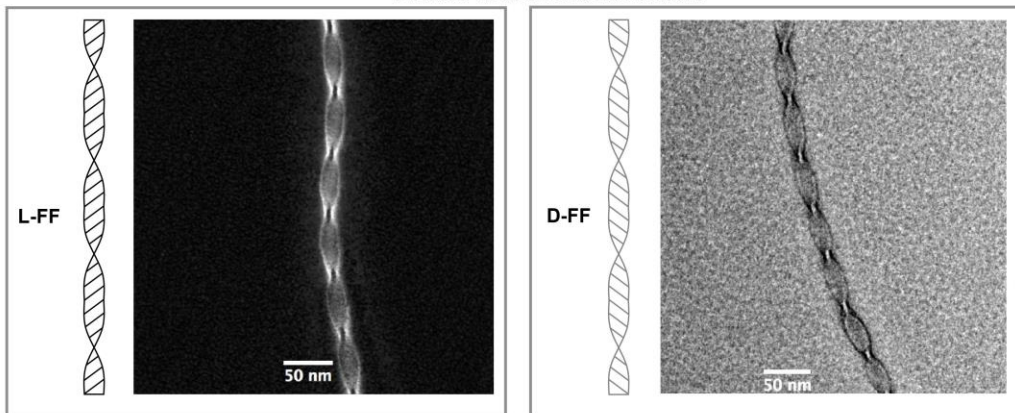
3 Hydrogen-bonding pattern and secondary structures of the networks were characterized
4 by circular dichroism (CD) spectroscopy. For L-VV and D-VV ensembles, β -sheet
5 secondary structure was detected by fingerprint bands at 221 nm and 200 nm (Figure 1B)
6 corresponding to $n-\pi^*$ and $\pi-\pi^*$ transitions, respectively.⁴⁰ CD spectra of L-FF and D-FF
7 ensembles were more complicated, presumably a combination of absorptions of β -sheet
8 motif and phenylalanine residues. In both cases, the CD spectra of L-form and D-form
9 peptide nanofibers were mirror images of one another due to their opposite chirality. In
10 the case of phenylalanine-containing L-FF and D-FF ensembles, a deviation was
11 observed from perfect β -sheet structure because $\pi-\pi$ interactions between aromatic side
12 chains caused building blocks to assemble not with a 90° angle to the elongation axis, as
13 with L-VV and D-VV, but with a narrower angle that rotated and twisted across the
14 length of the fiber. This rotation in conformation has been reported as a twisted β -sheet
15 secondary structure.³⁶ In twisted assemblies, relatively weaker hydrogen bonds are
16 formed due to the increases in bond length. Therefore, a red shift was observed in the CD
17 spectrum (Figure 1B). To get an estimate on contribution of phenylalanine residues,
18 spectra of L-VV and D-VV ensembles were subtracted from spectra of L-FF and D-FF
19 ensembles, respectively. CD difference spectra (Figure S5) are indicative of the
20 contribution of the phenylalanine residues to higher energy transitions ($n-\pi^*$ and $\pi-\pi^*$)
21 of the amide group. The CD difference spectrum between L-isomer ensembles showed
22 two positive peaks at 222 nm and 208 nm and a negative peak at 198 nm, which resemble
23 CD spectrum of N-acetyl-L-phenylalanine amide.⁴¹

A

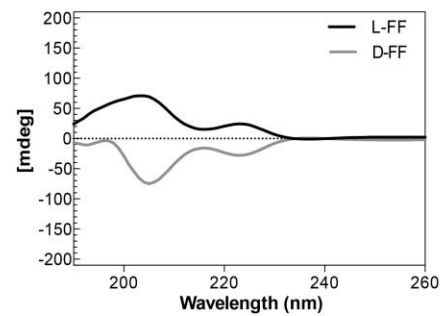
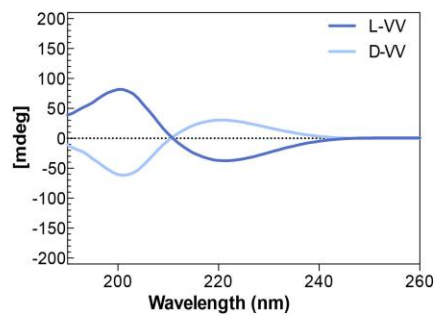
Cylindrical Nanofibers



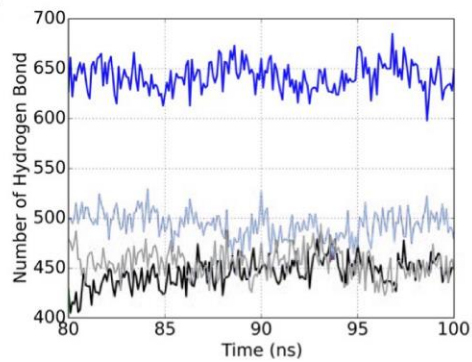
Twisted Ribbon-like Nanofibers



B



C



D

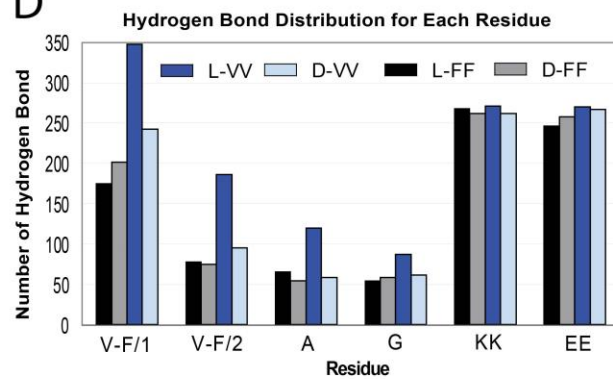


Figure 1. Structural and morphological characterization, and molecular dynamics simulations of peptide nanofibers; (A) Schematic representations of peptide nanofibers and their corresponding STEM images, scale bar=100 nm (L-VV and D-VV), scale bar=50 nm (L-FF and D-FF). (B) Circular dichroism spectra of peptide nanofibers. (C, D) Molecular dynamics analysis of peptide nanofibers. Hydrogen bonds are determined by the following criteria: when distance between donor and acceptor atoms are lower than 3.5 Å, and donor-hydrogen-acceptor angle is greater than 150°. (C) Time series of hydrogen bond number for the last 20 ns of the simulations. (D) Number of hydrogen bonds that each residue forms: V-F/1 denotes the first valine or phenylalanine (numbering starts from the lipophilic tail) on the corresponding PA; V-F/2 denotes second V or F, KK and EE denote merged results for two lysine and two glutamic acid residues, respectively. L-VV: Lauryl-VVAGEE-Am / Lauryl-VVAGKK-Am, D-VV: Lauryl-vvaGee-Am / Lauryl-vvaGkk-Am, L-FF: Lauryl-FFAGEE-Am / Lauryl-FFAGKK-Am, D-FF: Lauryl-ffaGee-Am / Lauryl-ffaGkk-Am.

All-atom explicit solvent classical molecular dynamics simulations were performed to investigate the molecular organization of the PAs at the atomic level (Figure S8-S11). Time series of the non-bonded interactions within peptide structures are shown in Figure S9A. Total non-bonded energies of L-FF and D-FF peptide nanofibers are similar to each other and higher than D-VV and L-VV nanofibers. On the other hand, L-VV nanofibers have lower non-bonded interaction energy than their D-VV counterparts. When only van der Waals interactions are considered, phenylalanine- and valine-containing PAs show different profiles. L-VV and D-VV nanofibers have lower energies, whereas L-FF and D-FF nanofibers have more van der Waals interactions. To elucidate this behavior, we calculated only the non-bonded interaction energies between atom pairs comprised of the side-chains of phenylalanine and valine amino acids (Figure S9B). We observed a high difference in the van der Waals interaction energy between peptide nanofibers having phenylalanine and valine residues. This suggests that van der Waals energy difference between nanofibers containing phenylalanine and valine residues is due to high repulsion between phenylalanine side-chain atoms. It is also important to note that even though phenylalanine residues form favorable electrostatic interactions compared to valine residues, and the total non-bonded interaction energy is dominated by van der Waals interactions (Figure S9). High van der Waals repulsion between phenylalanine residues plays a critical role in the formation of twisted nanofibers. Even though the time scale of the simulations is not long enough to observe twisting in phenylalanine-containing

nanofibers, energy analysis shows the driving force for formation of twisted nanofibers. Hydrogen bond analysis for the peptide nanofibers (Figure 1C) shows the number of unique inter-peptide H-bond in each nanofiber as a function of simulation time. Phenylalanine-containing nanofibers have the lowest number of H-bonds; the number of H-bonds in D-FF and L-FF nanofibers fluctuates around 450. In contrast, the H-bond number in valine-containing peptide nanofibers increases up to 500 in D-VV and 650 in L-VV nanofibers. This difference in H-bond number between valine and phenylalanine-containing nanofibers suggests that bulky aromatic phenylalanine side-chains could limit the H-bond formation ability of phenylalanine-containing nanofibers. Interestingly, the number of H-bonds in D-VV is much lower than the number of H-bonds in L-VV nanofiber. The contribution of each amino acid to the H-bond number is shown in Figure 1D. Individual contribution of amino acids in phenylalanine-containing nanofibers does not differ considerably in L- and D- enantiomers. However, all amino acids except K and E residues in valine-valine containing nanofibers have more H-bonds in L-form than in D-form. The two valine residues contribute substantially to the H-bond number difference between L- and D-forms of valine-containing PAs. Therefore, we investigated conformational and dynamic behavior of valine residues in L- and D-form to understand the H-bond number differences in valine residues. Valine residues sterically hinder H-bond formation in D-form as shown in radial distribution function (RDF) analysis. Figure S10 demonstrates atom-pair distribution as a function of pair-distance for the atom-pairs between valine side-chain gamma carbons and valine backbone oxygen, and the atom-pairs between valine side-chain gamma carbons and hydrogen atom bound to amide nitrogen. In these plots, the valine side-chain gamma carbons are closer to the valine backbone in D-form compared to the L-form. This result suggests that side-chain steric hindrance plays a role in hampering H-bond formation in D- amino acid-containing nanofibers. Figure S11 shows Ramachandran plots for valine residues in L- and D-nanofibers. Dihedral angles for the first valine residue in L-form populated around $\phi = -110$, $\psi = -135$, which is close to the β -sheet region center (a study done by Hovmöller *et al.* on the analysis of protein structures reports that backbone dihedrals of valine amino acid for β -sheet forming conformations populate around the center where $\phi = -117.7$ and $\psi = 127.8$ for parallel beta strands, and $\phi = -121.2$ and $\psi = 132.5$ for antiparallel

1 beta strands⁴²). However, dihedral angle population for the first valine in D-form shifts to
2 the center where phi and psi angles around -90 and -125 respectively. Dihedrals of the
3 second valine in L-form nanofibers are not localized at a certain region; they can sample
4 both regions near the ideal beta sheet region and regions where phi and psi are around -
5 140 degrees. On the other hand, dihedral angle population for the second valine in D-
6 form PAs shifts towards the upper side of the Ramachandran plot. According to
7 Hovmöller *et al.*, for conformations that form random coil structures, valine dihedrals
8 shift towards higher phi and lower psi angles.⁴² Hence, the dihedral angle population shift
9 observed in our calculations for D-amino acid-containing nanofibers could imply that
10 backbone conformation in D-amino acids deviates from the beta sheet region towards
11 random coils. Hence, this could affect the H-bond forming ability of D-valine residues in
12 PAs that form nanofibers.

13 Oscillatory rheology measurements were performed for mechanical analysis of the bulk
14 gel. Time sweep, frequency sweep and strain sweep rheology analysis were conducted in
15 order to investigate the gel formation mechanics and viscoelastic properties of the peptide
16 nanofiber networks (Figure S12). Storage moduli of all nanofiber networks were found to
17 be higher than their loss moduli suggesting that the materials are hydrogels. In addition,
18 we found that cylindrical nanofiber networks have higher elastic moduli than twisted
19 ribbon-like nanofiber networks. On the other hand, we did not detect any difference in
20 elastic moduli of nanofibers between L- and D- forms. In addition, we performed SEM
21 analyses, which showed that the nanofiber network morphology was similar for all bulk
22 hydrogel groups (Figure S22). For nanomechanical characterization of peptide
23 nanofibers, elastic behavior of peptide nanofibers was investigated by atomic force
24 microscopy (AFM) measurements (Figures S13 and S14). Force mapping measurements
25 were employed on nanofibers and nanobundles, and elastic modulus values were
26 calculated by fitting approach curves using the Hertz model in MATLAB (Figure S13).
27 Both L-form of cylindrical and twisted ribbon-like nanofibers had significantly higher
28 elastic moduli than their D-forms. L-VV nanofibers had the highest stiffness at 45.62
29 MPa, D-VV had 15.03 MPa, L-FF had 32.04 MPa and D-FF had 10.03 MPa.
30 Interestingly, we observed that the difference in elastic moduli of L- and D- form of VV-

nanofibers is consistent with the H-bond density according to molecular dynamic simulations.

Peptide nanofibers provide a biocompatible environment for cell culture

Cellular viability, adhesion, proliferation, and spreading were evaluated qualitatively and quantitatively prior to testing the effects of nanofiber morphology and chirality on cellular behavior. Viability analysis showed that all peptide nanofibers were biocompatible and conducive to the growth and proliferation of stem cells through 3 culture days (Figure S15A). Live/Dead assay also showed that few to no dead cells were present on all scaffolds, which supports the results of Alamar Blue assay and demonstrates that these peptide nanofibers are highly biocompatible (Figure S16). In addition, Alamar Blue results indicated that the morphology and chirality of the peptide nanofibers do not affect the metabolic activity of cells.

When cells are exposed to a new microenvironment, cellular processes are initially altered to mediate substrate adhesion prior to the resumption of regular metabolic pathways. At this initial step, the primary role of the nanostructure scaffold is to provide a suitable set of signals for cell attachment. Adhesion analysis of peptide nanofiber structures showed that all peptide nanofibers facilitated the initial attachment of stem cells after 1 h and 5 h of culturing (Figure S15B, C). When compared to tissue culture plate (TCP), cells were found to immediately attach to peptide nanofiber surfaces within the first few hours (1-5 h) of culture, and no significant differences were observed in the initial attachment of cells among different morphological and chiral groups.

In addition, we analyzed collagen adsorption on the peptide nanofibers to examine whether there is any differential effect of these nanofibers on the matrix protein adsorption. We found that there was no specific collagen binding on none of the nanofiber groups (Figure S21). We did not measure specific absorbance values different from blank controls (only peptide nanofibers without collagen addition), which indicated that there was no specific binding of collagen and there was no difference among peptide nanofiber groups. When there is specific epitope, on the other hand, there should be specific binding that should cause the significant increase in the absorbance that we measured in our previous studies by using same optimized protocol.^{66,67} This result showed that peptide nanofibers can adsorb proteins but interactions among them are

1 short-term transitional interactions. If there is no specific binding epitope on the peptide
2 amphiphile molecules, any kind of protein (such as ECM protein, growth factors, or
3 antibodies) can interact non-covalently with peptide nanofibers regardless of their
4 chirality or morphology. However, these interactions are not permanent and cellular
5 adhesion is not being interrupted by these adsorptions for our case. In this respect,
6 although all of the peptide nanofibers have similar binding capacity for collagen
7 adsorption, we observed different level of cellular responses among peptide nanofiber
8 groups, which we concluded that differences in cellular responses were resulted via
9 different mechanotransduction pathways directly depending on peptide nanofiber-cell
10 interactions. Similarly to this observation, nanoscale topography was shown to regulate
11 collective cell function through cell adaptation mechanism largely independent of
12 adsorbed proteins.⁶⁸

13 In addition to stem cells, an adherent primary human cell line (HUVECs) and human
14 fibroblasts (hFibs) were also analyzed to determine the effect of peptide nanofibers
15 morphology and chirality on cellular responses, and both cell types were found to readily
16 adhere to peptide scaffolds (Figure S17A, B). While HUVECs adhered significantly less
17 on L-form of twisted-ribbon-like nanofibers (L-FF) among other groups, there were no
18 significant differences between the groups for the adhesion of hFib cells (Figure S17B).
19 The adhesion results, therefore, show the number of cells that were able to initially
20 establish themselves on the peptide nanostructures.

21 **Cellular proliferation and spreading is mediated by nanofiber morphology and** 22 **chirality**

23 Following cellular adhesion, a bioactive scaffold should ideally provide the necessary
24 signals for activating cellular processes such as proliferation, spreading and
25 differentiation. According to proliferation analysis results (Figure 2A), cells
26 demonstrated different proliferation rates on different peptide nanofibers, suggesting that
27 nanofiber morphology and chirality play an important role in mediating nanofiber-cell
28 interactions. Proliferation of rMSCs was tracked for 5 days, and both L- and D- forms of
29 the twisted ribbon-like nanofibers (FF) were found to elicit significantly lower
30 proliferation of rMSCs during 5 days of incubation. After 1 day of culture, cells
31 proliferated on L- and D- forms of the cylindrical nanofibers (VV-PA) to a much greater

1 extent than FF-nanofiber and TCP groups. Moreover, the importance of chiral differences
2 for cylindrical nanofibers was seen even after 3 days of culture, where proliferation rates
3 of rMSCs on L-VV decreased much more than D-VV. After 5 days, proliferation rates of
4 rMSCs cultured on the L- and D- forms of VV-nanofibers had decreased, but were still
5 higher than FF-nanofiber groups. Confluence is a likely reason for this effect, since L-VV
6 and D-VV cells would stop proliferating through contact inhibition after an early increase
7 in their population. In contrast, L-FF and D-FF do not rapidly crowd the well plate and
8 can sustain their growth for a longer period of time but do not increase the proliferation,
9 which is potentially due to regulation of cell differentiation by different morphological
10 signals than VV- nanofibers, which was also evident in chondrogenesis potential of cells
11 on FF-nanofibers. In addition, studies on stem cells also showed that for particular
12 differentiation lineages, proliferation of stem cells were decreased⁴³. Overall, peptide
13 nanofibers were found to strongly alter cellular processes even after short-term (24 h)
14 culture, and proliferation on twisted ribbon-like nanofibers (FF-PAs) was limited
15 compared to cylindrical nanofibers (VV-PAs).

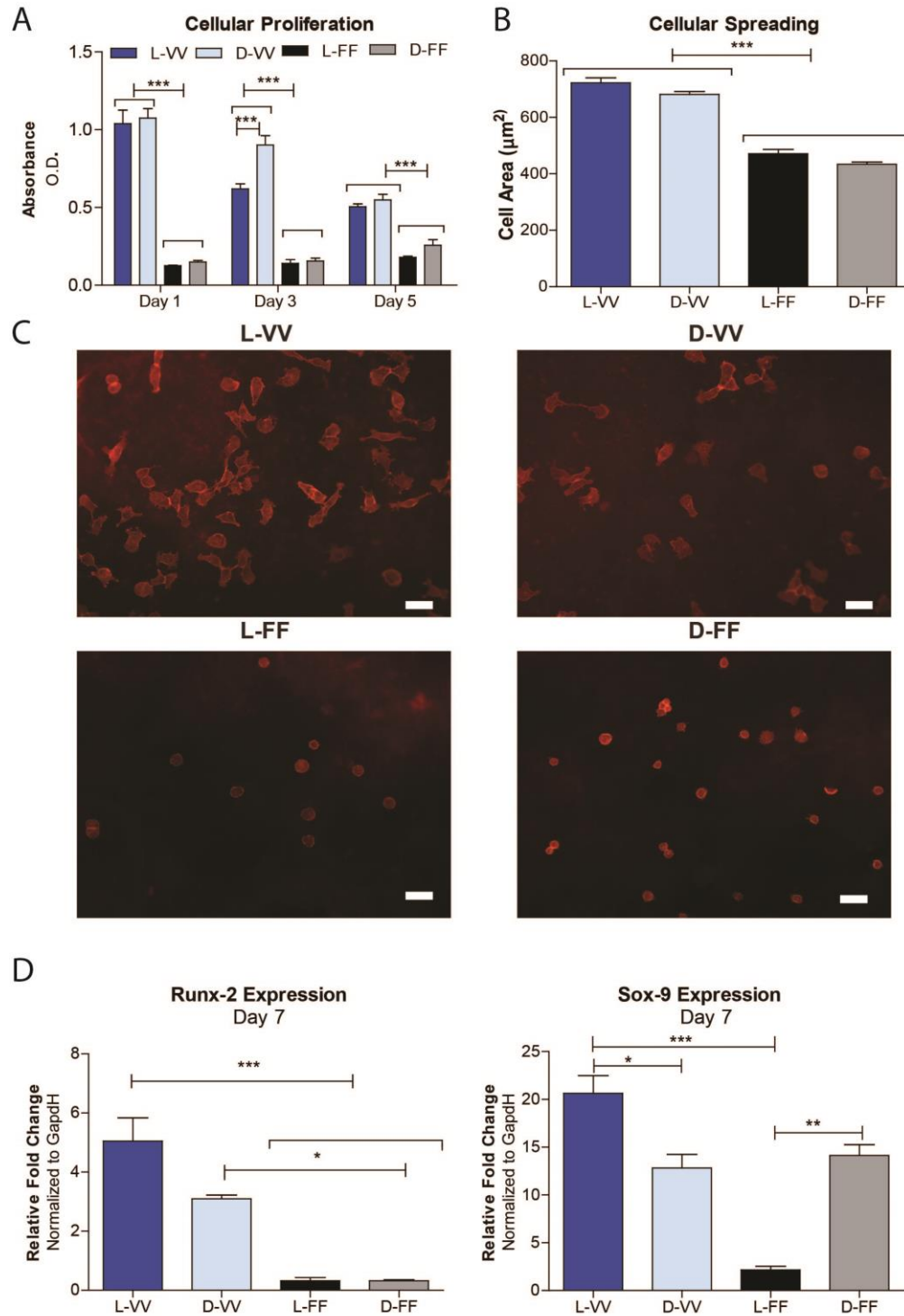


Figure 2. Cellular proliferation, spreading, and differentiation analyses of MSCs on peptide nanofibers. (A) Cellular proliferation measurement of MSCs by BrdU assay through 5 days of culture (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-way ANOVA with Bonferroni's post-hoc test, mean \pm s.e.m.), (B) Quantification of cellular spreading measurements of MSCs 24 h after seeding on peptide nanofibers, obtained by Image J

analysis of cells stained with phalloidine and imaged by fluorescence microscopy (n>50 cell per group (*p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Bonferroni's post-hoc test, mean \pm s.e.m.). (C) Representative fluorescent images of MSCs 24 h after seeding on peptide nanofibers, scale bar = 50 μ m. (D) Osteogenic and chondrogenic differentiation analyses by qRT-PCR. Expression of Runx-2 and Sox-9 were quantified on day 7 (*p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Bonferroni's post-hoc test, mean \pm s.e.m.)

After analyzing the collective response of cell cultures to different nanofibrous morphologies and chirality, cellular areas were measured to investigate individual cellular responses (Figure 2B and 2C). Although neighboring cells and cellular confluence may affect the spreading of cells, each cell spreads individually through its own receptors and specific receptor-ligand interactions.⁴⁴ Cells were seeded on all peptide nanofiber groups at the same cell concentration to avoid any initial confluency differences. We had already observed that the cells all adhered similarly (at the time of initial attachment of cells through 1-5 h) according to cellular adhesion analysis. The rMSCs cultured on L-and D- form of twisted ribbon-like nanofibers (FF-PA) exhibited significantly less cell spreading than those cultured on cylindrical nanofibers (VV-PA). In addition to rMSCs, the spreading of HUVECs and hFibs was also evaluated (Figure S16C). Among peptide nanofibers with different morphology and chirality, HUVECs on the L-form of nanofibers (L-VV) exhibited less spreading than the D-form nanofibers (D-VV). As with other cell lines, the L-FF twisted ribbons limited the spreading of hFibs to a greater extent than the D-FF nanostructures, while the D-form of VV-nanofibers was much more inhibitory than the L-form for VV-nanofibers. As such, both the morphology of nanofibers and their chirality had distinctive effects on cellular responses. Twisted ribbon-like nanofibers exhibited a more pronounced restrictive effect on cellular spreading in the L-form; whereas cylindrical nanofibers were more inhibitory in their D-form, especially for fibroblasts. Although we observed similar mechanotransductive responses on peptide nanofibers for different cell types, to elaborate on the inherent effect of designed peptide nanofibers for future clinical applications, we focused on stem cells for the analysis of cell-material interactions at the molecular level for further experiments.

Effects of peptide nanofibers with different morphologies on the osteochondrogenic differentiation of MSCs

When cells are exposed to a microenvironment, their morphology, spreading area, and proliferation rate are altered, resulting in the activation of specific response mechanisms that directly affect the organism at cell and tissue levels.⁴⁵ A thorough understanding of cell-materials interactions is essential to regulate these responses. These effects are also important for the clinical applicability of stem cells, which are being utilized for the treatment of various diseases and biomedical applications. The primary advantage of stem cells is their ability to differentiate into multiple lineages, which can be mediated through their cultivation on peptide scaffolds or other biomaterials. Thus, the differentiation potential of rMSCs were analyzed on peptide nanofibers with different morphology and chirality. Mesenchyme-originated stem cells may differentiate into osteogenic, chondrogenic, and adipogenic lineages, and the initial commitment of cells to differentiate into the osteogenic and chondrogenic lineages was investigated by qRT-PCR analysis on day 7. Runx-2, a transcriptional factor, which is expressed during the osteogenic differentiation of stem cells to activate the synthesis of bone specific proteins, was found to be upregulated on both L- and D-forms of cylindrical nanofibers (L-VV and D-VV), with L-VV nanofibers exhibiting a more pronounced effect on Runx-2 expression than the D-form (Figure 2D). In contrast, twisted ribbon-like nanofibers (L-FF and D-FF) significantly inhibited osteogenic differentiation of MSCs. These twisted ribbon-like nanofibers (L-FF and D-FF) instead increased the chondrogenesis of stem cells, as shown by increased in Sox-9 expression, which was especially significant on D-FF nanofibers (Figures 2D and S18).

Scaffold-mediated structural and chiral regulation of mechanotransduction

Since both twisted ribbon-like and cylindrical nanofiber systems presented the same amino acid sequences on their periphery, the differential bioactivity of these nanofibers was analyzed to investigate the effects of morphology and chirality on mechanotransduction of cells. Protein expression levels of key regulators for mechanotransduction-specific molecular pathways were investigated by Western blot analysis to further evaluate the effect of cell-material interactions of rMSCs on peptide nanofiber scaffolds. Cellular mechanotransduction is initiated by the binding of stretch

1 receptors to nanofibers, which activates a protein cascade to convert mechanical
2 information into biochemical signals.⁴⁵ Integrin $\beta 1$ receptor is a key player in the early
3 steps of this process and forms a heterodimeric complex with several integrin α receptors
4 to bind fibrous proteins of the ECM and initiate the formation of focal adhesion
5 complexes. Western blotting analysis of integrin $\beta 1$ showed that its expression is
6 upregulated on the L-forms of cylindrical (L-VV) and twisted ribbon-like nanofibers (L-
7 FF) compared to the D-forms (Figure 3A). Between the two distinct morphologies,
8 cylindrical nanofibers (VV-) significantly increased the expression of integrin $\beta 1$
9 compared to twisted ribbon-like nanofibers. In addition, L-VV also had a greater capacity
10 to induce integrin $\beta 1$ expression in MSCs compared to D-VV, suggesting the importance
11 of chirality for cell-ECM interactions.

12 While integrin receptors provide the initial interaction of peptide nanofibers with cells,
13 the transduction of this information entails a complex signaling cascade (Figure 3B). The
14 phosphorylation of ERK and MEK is an integral step in this process and was investigated
15 by Western blotting analysis. Significant differences were observed in the expression of
16 MEK in MSCs on peptide nanofibers with different morphologies; in particular, rMSCs
17 on L-form nanofibers invariably had higher MEK levels than their D-form counterparts,
18 and cylindrical nanofibers (L-VV) had increased MEK expression compared to twisted
19 ribbon (L-FF) nanofibers (Figure 3D). In addition, MSCs cultured on peptide nanofibers
20 all expressed phosphorylated and activated ERK, but at different levels (Figure 3C; non-
21 activated ERK was not detected in the groups and is not shown). The pERK expression
22 patterns were similar to integrin $\beta 1$, as L-forms of both VV- and FF-nanofibers had
23 enhanced pERK expression, and cylindrical nanofibers enhanced phosphorylated ERK
24 levels to a greater extent than twisted ribbon-like nanofibers.

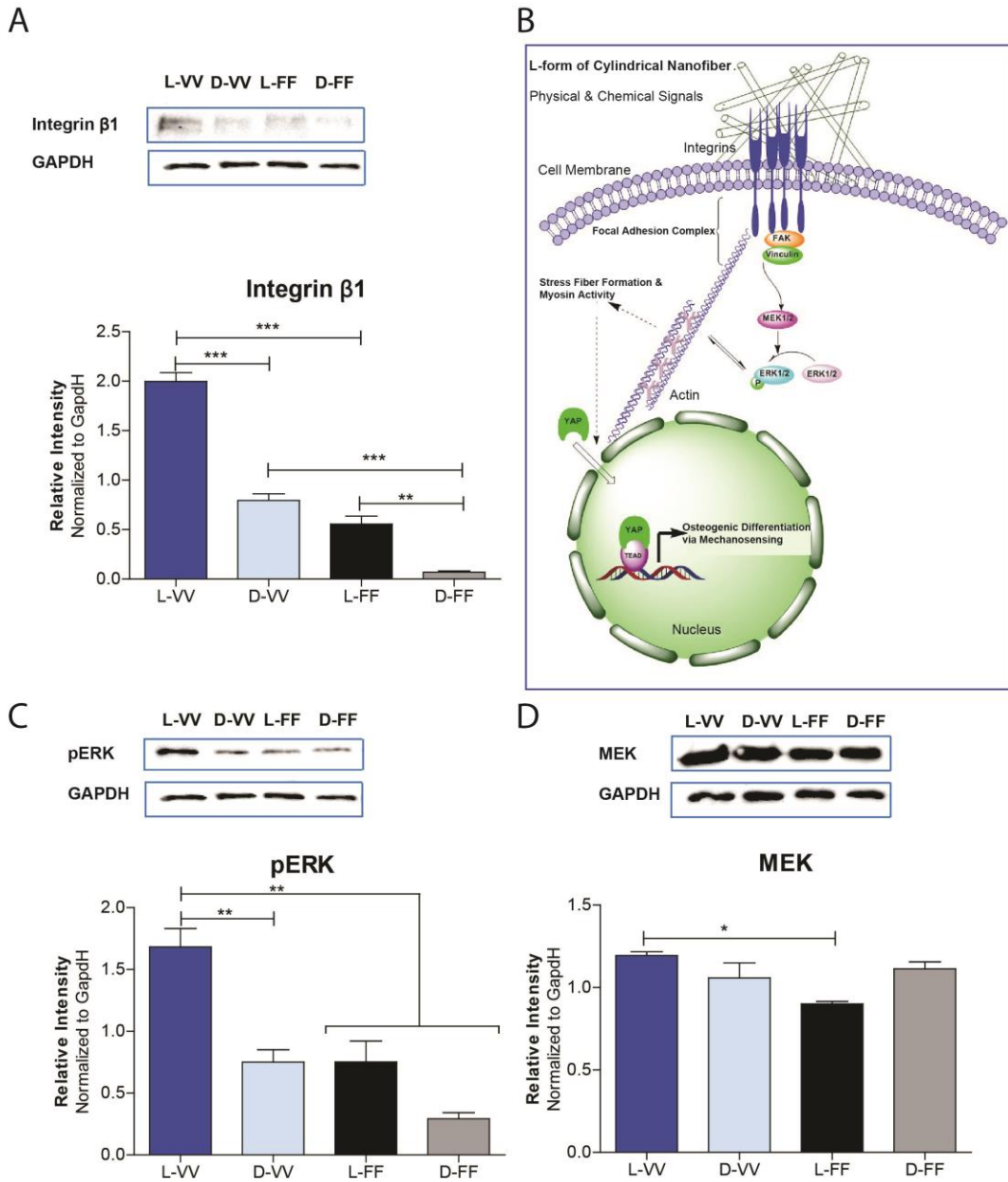


Figure 3. Signaling pathway analyses of cellular mechanotransduction; (A) Integrin $\beta 1$ expression on peptide nanofibers with different morphology and chirality, as analyzed by Western blotting (** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Bonferroni's post-hoc test, mean \pm s.e.m.); (B) Schematic representation of the signaling pathway activated in MSCs cultured on the L-form of cylindrical nanofibers; (C) pERK expression on peptide nanofibers, as analyzed by Western blotting (** $p < 0.01$ by one-way ANOVA with Bonferroni's post-hoc test, mean \pm s.e.m.); (D) MEK expression on peptide nanofibers, as analyzed by Western blotting (* $p < 0.05$ by one-way ANOVA with Bonferroni's post-hoc test, mean \pm s.e.m.)

Vinculin expression was analyzed by both flow cytometry and confocal microscopy to determine the changes in focal adhesion point formation on peptide scaffolds (Figures 4 and S19B). The localization of vinculin proteins is essential for the analysis of focal adhesion complexes, since vinculin plays an essential role in strengthening the adhesion complex by bearing force loads during the adhesion process.^{44, 46} According to the confocal microscopy analysis, actin filaments were organized into well-defined stress fibers in cells on L-VV nanofibers (Figure S20). In addition, vinculin was strongly expressed on the protrusions of the cell membrane where focal adhesion complexes are formed. In contrast, on D-VV nanofibers, cells did not form stress fibers, vinculin proteins were observed in the cytosol, and the cells were not observed to develop long filopodia. Similarly, on twisted ribbon-like nanofibers (L-FF and D-FF), cells failed to develop stress fibers or form cellular protrusions; instead, they retained a spherical morphology with few adhesion points.

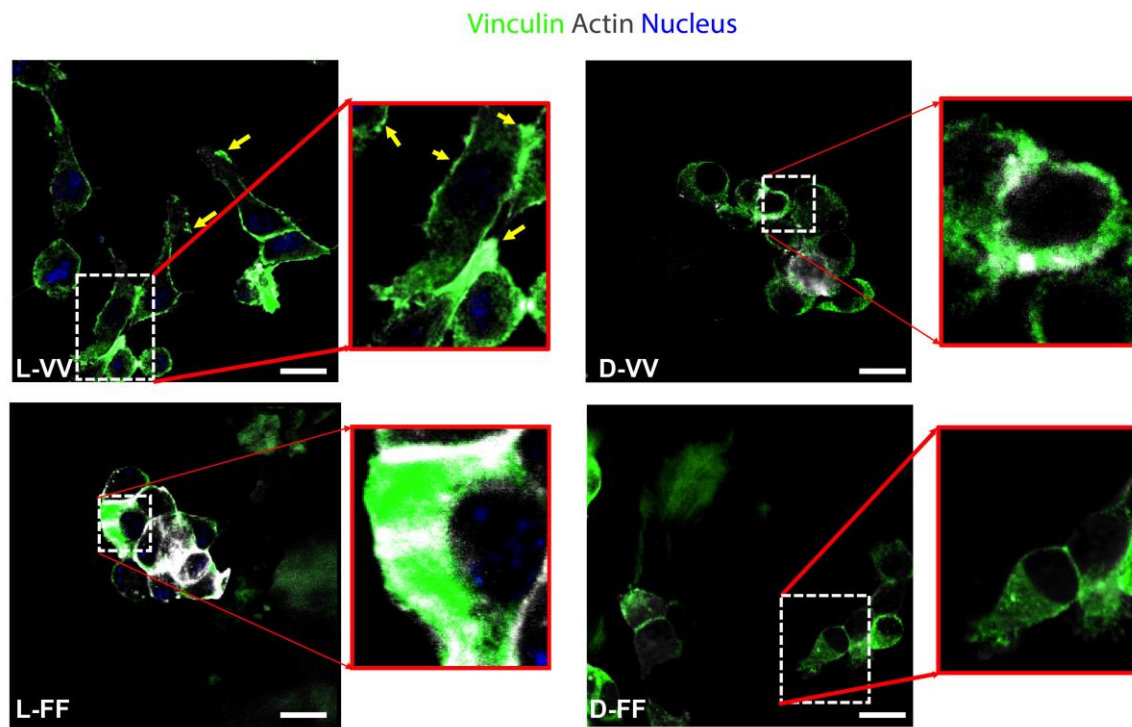


Figure 4. Focal adhesion complex analyses of MSCs on peptide nanofibers with different morphology and chirality; Confocal images of MSCs on peptide nanofibers stained for the visualization of actin stress fibers (grey), vinculin (green), and nuclei (blue), scale bars=20 μm .)

1

2 To demonstrate the molecular effect of peptide nanofiber morphology and chirality on
3 cellular processes, the expression of YAP (Yes-associated protein, which is a
4 transcriptional coactivator involved in organ growth) was assayed by
5 immunocytochemical analyses. YAP is a transcription coactivator that regulates many
6 cellular processes by shuttling between the nucleus and cytosol and interacting with
7 transcription factors to inhibit or activate the transcription process.⁴⁷ The mechanical
8 regulation of YAP activity involves the activation of F-actin capping/severing proteins
9 and the formation of stress fibers, which function as a mechanical rheostat in the
10 mechanotransduction of cells.^{47, 48} When YAP proteins are active, they localize into the
11 nucleus and function as transcriptional co-activators; however, when they are inactive,
12 they remain in the cytosol (Figure 5B). According to confocal analyses, cells having
13 nuclear YAP localization were quantified and the number of cells that had YAP in their
14 nuclei was found to be significantly higher in L-VV group compared to other groups,
15 suggesting that the activation of YAP occurs within 24 h of cell seeding on L-VV
16 nanofibers (Figure 5A). However, most of the MSCs cultured on the D-form of this
17 cylindrical nanofiber (D-VV) had still inactive YAP proteins in their cytosol after 24 h
18 incubation. Similarly, most of the cells on twisted ribbon-like nanofibers (both L-and D-
19 from of FF- nanofibers) had inactive YAP proteins in their cytosols (Figure 5A & 5B).
20 Thus, the influence of supramolecular chirality on cells is decisive on even their gene-
21 level regulation, and introduces an additional level of complexity to the structural and
22 mechanical effects of nanofibers.

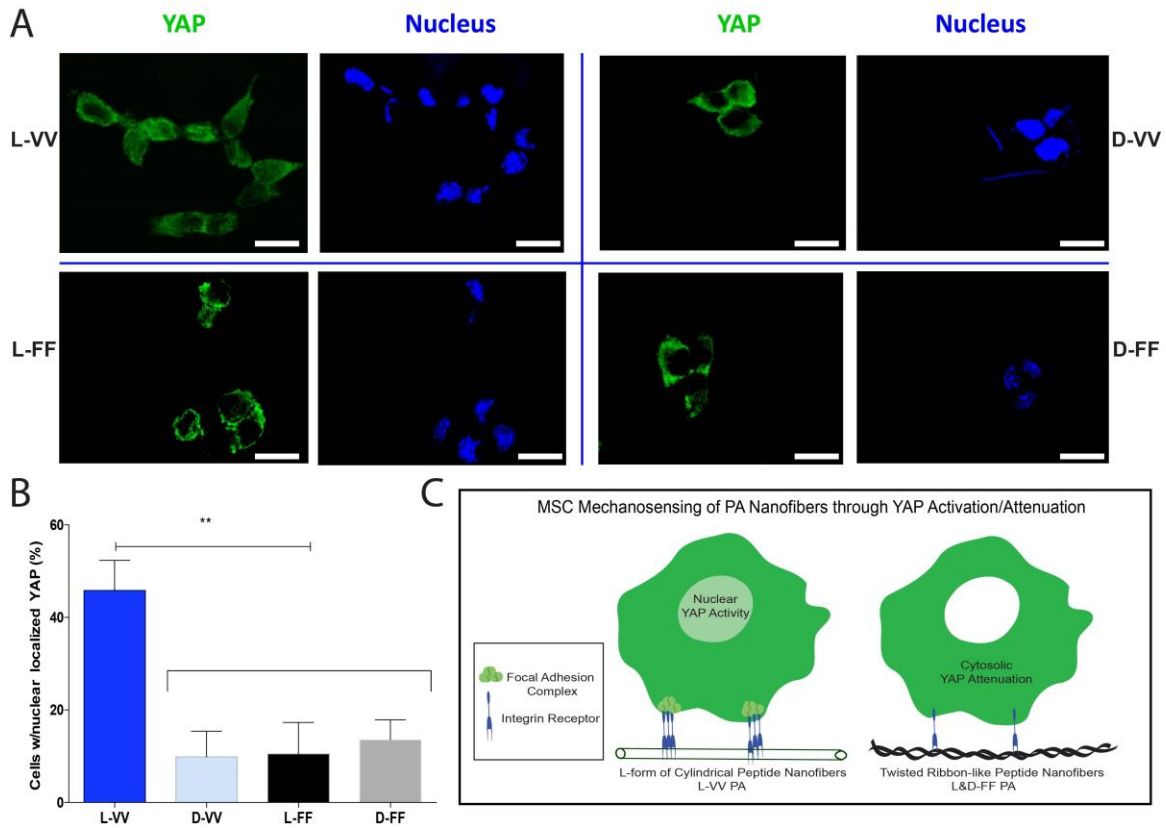


Figure 5. Cellular mechanosensing of peptide nanofibers with different morphology and chiralities through YAP activation/attenuation; (A) Confocal microscope images of MSCs stained for the visualization of YAP proteins (green) and nuclei (blue), scale bars=20 μ m; (B) Cells with nuclear YAP localization were quantified from the confocal images, which shown as percent of cells in the all counted cells (** $p < 0.01$ by one-way ANOVA with Bonferroni's post-hoc test, mean \pm s.e.m.) (C) Schematic representation of MSC response on different PA nanofibers through activation/attenuation of YAP protein into the nucleus/cytosol.

DISCUSSION

In addition to their successful use in medical applications, supramolecular nanostructures present a great opportunity to study cell-materials interactions by emulating the native ECM.^{13, 15} Limitations in the in-depth analysis of cell-material interactions at the molecular level complicate potential investigations into the mechanical regulation of cells through morphological signals provided by the extracellular environment. Here we designed and synthesized a series of nanofiber scaffolds with well-defined structural properties, and used them to investigate the effects of fiber morphology and chirality on cellular behavior. Each peptide nanostructure was synthesized homogenously and showed

1 no variance in physical and chemical features. L-VV and D-VV peptide nanofibers
2 exhibited cylindrical morphology while L-FF and D-FF peptide nanofibers had a twisted
3 ribbon-like morphology, as characterized by TEM analyses. Their chiral signatures were
4 further analyzed and confirmed by CD measurements.

5 Peptides and proteins regulate their chirality through the self-assembly of their amino
6 acid residues.⁴⁹ Therefore, different supramolecular systems with distinct structural
7 features can be developed by altering assembly dynamics through the use of L- or D-
8 enantiomers of amino acids.⁵⁰ In addition, self-assembly of nanomaterials can be
9 reconfigured, and unique mechanical properties and complex topologies can be
10 developed by controlling the interfacial tension of chiral materials.⁵¹ Here we showed L-
11 and D- forms of morphologically different nanofibers and observed that differences in
12 chirality and morphology can result in the activation or repression of distinctive cellular
13 processes. In rheology measurements, all peptide nanofiber networks displayed elastic
14 solid-like behavior with high water content, and we did not detect any difference in
15 elastic moduli of nanofibers between L- and D- forms. On the other hand, significant
16 differences were observed in the elastic moduli of the L- and D- forms of peptide
17 nanofibers through the AFM measurement of nanofibers and nanobundles (Figures S13
18 and S14). All nanofibers have megapascal-level elastic modulus values in aqueous
19 environment, which emulates the conditions experienced by cells. Although the effect of
20 stiffness on cellular behavior is well-documented in the literature, such studies generally
21 focus on the mechanical properties of bulk gels.⁵² In this study, AFM force map analysis
22 provided deep insights for the elastic behavior of nanofibers, which were not detected by
23 rheological analysis. When considering the concentration difference between two
24 measurements, rheology provided bulk macro-scale measurement depending on the
25 highly-concentrated nanofiber network properties where molecular interactions could not
26 be detected precisely. On the other hand, AFM provided nanoscale mechanical analyses,
27 which is the scale at which cellular interactions occur. In addition, it was previously
28 indicated that rather than bulk stiffness, mechanical feedback gathered from the
29 interaction through collagen fibrils and integrin receptors has profound effect on the stem
30 cell behavior.⁵³ Similarly, we found that both morphology and chirality of nanofibers had
31 profound effects on their mechanical stiffness: L-VV nanofibers formed more stiff fibrils

1 than D-VV and FF-peptide nanofibers, which is relevant to their biological activity that
2 we observed due to the well-known impact of stiffness on the initiation of osteochondral
3 differentiation.^{54, 55} As observed in the results of molecular dynamics simulations, D-VV
4 had lower hydrogen bonding density than the L-form due to side-chain steric hindrance,
5 which affects the rigidity of nanofiber formation. Similarly, twisted ribbon-like
6 nanofibers had lower hydrogen bonding density according to molecular dynamics
7 simulations, and the red shift in the CD spectrum of FF containing nanofibers is
8 indicative of weaker hydrogen bond formation. L-VV, on the other hand, has a much
9 higher H-bond density, which allows the rigid packing of structure and results in stiffer
10 nanofiber formation, while lower H-bond density and weaker bond formation cause
11 looser packing of PAs during self-assembly of nanofibers. The crucial role of rigid
12 backbone in viscoelastic properties was also shown in a previous study⁵⁶. Hence, the
13 nanofibers with different morphology and chirality were developed through strictly
14 controlled modifications in the design of peptide amphiphile molecules.

15 In this study, stem cells exhibited differential responses to different morphological and
16 chiral signals. Osteogenesis and cell spreading were strongly stimulated on the L-form of
17 cylindrical nanofibers (L-VV) compared to D-VV, L-FF and D-FF groups. This result is
18 consistent with the previous reports, as a high spread area has been reported to promote
19 the osteogenic differentiation of MSCs.⁵⁷ The spreading of cells provides them with
20 higher contractility through increased stress fiber formation, which increases their
21 response to soluble factors such as autocrine/paracrine Wnt signals, as well as osteogenic
22 media supplements⁵⁸. In addition, Sox-9 expression was also detected in FF-groups. Sox-
23 9 is indeed expressed during both osteogenic and chondrogenic differentiation at the
24 earlier phase of mesenchymal differentiation; consequently, Sox-9 expression in the
25 presence of osteogenic markers (e.g. Runx-2) is considered to be an indicator of
26 osteogenesis, while Sox-9 in the absence of osteogenic gene expression is a marker of
27 chondrogenesis. Indeed, in the study of Akiyama et.al., osteo-chondroprogenitor cells, as
28 well as progenitors in a variety of tissues, were shown to be derived from Sox-9-
29 expressing precursors during mouse embryogenesis, and inactivation of Sox-9 resulted in
30 prevention of mature osteoblast formation⁵⁹. Hence, we also showed both L- and D- form
31 of cylindrical nanofibers increased the Sox-9 expression at mRNA level- where L-VV

1 had more pronounced effect. On the other hand, twisted ribbon-like nanofibers increased
2 the Sox-9 expression in the absence of Runx-2 and promoted the chondrogenesis of
3 MSCs. In fact, both L-and D- forms of the twisted ribbon nanofibers greatly reduced
4 cellular proliferation as well as cellular spreading, which resulted in substantially lower
5 osteogenesis. This restriction of cellular spreading was also seen for HUVECs and hFib
6 cells cultured on the L-form of twisted ribbon-like nanofibers. Interestingly, the
7 morphological effect on cellular spreading was more profoundly seen on the L-form of
8 twisted ribbon-like nanofibers, which also indicates the importance of chiral signatures
9 for the regulation of cellular behavior. For cellular adhesion, differences among nanofiber
10 groups were only observed for HUVECs, which is probably due to this cell line's
11 inherent susceptibility toward physical changes in environment: endothelial cells are
12 required to stretch, withstand strong shear forces, and prevent the development of
13 atherosclerotic plaques and aberrant neovascularization in their native environment.⁶⁰
14 Therefore, the difference in the morphology of the nanofibers had a profound effect on
15 the adhesion of HUVECs.

16 The minimal cellular proliferation and the spherical, non-adhering morphology of rMSCs
17 on FF-PAs were conducive for chondrogenic differentiation, which was further enhanced
18 when the cells were exposed to chondrogenic medium (Figure S18). The regulation of
19 cell spreading is an essential requirement for optimal cellular differentiation, and high
20 cell density have been reported to stimulate the chondrogenic differentiation of MSCs,⁵⁷
21 however, we observed scaffold-driven chondrogenesis at a relatively lower cellular
22 density on D-FF nanofibers. The combination of structural and morphological signals
23 from the nanofiber surface and soluble chondrogenic factors from the differentiation
24 medium further enhanced the chondrogenesis process on the D-form of ribbon-like
25 nanofibers. Interestingly, while ribbon-like nanofibers significantly inhibited
26 osteogenesis, they also strongly promoted chondrogenesis, especially in their D-form. As
27 such, peptide scaffold morphology can be used to control the differentiation of MSCs into
28 two closely related lineages. The spreading behavior of rMSCs on VV- and FF-
29 nanofibers is also consistent with previous research,^{57, 61} as the less-spread cells on
30 ribbon-like nanofibers differentiated into chondrogenic lineage, while the well-spread
31 cells on cylindrical nanofibers committed to osteogenesis.

1 To better understand the underlying cellular mechanisms of these distinct responses, we
2 further analyzed the specific components of the signaling pathways that are associated
3 with the cellular adhesion, migration, and differentiation of MSCs. Kilian *et al.*
4 previously showed that cell contractility, which is directly related to spreading of cells,
5 activates the ERK/JUN pathway.⁵⁸ We also observed an increase in p-ERK expression in
6 MSCs on L-VV nanofibers, and a significantly higher potential for osteogenesis through
7 the activation of ERK/MAPK pathways even on the first day of culture.^{62, 63} In addition,
8 in the MEK1-2 (MAP/ERK kinase 1-2) expressions of cells on L-VV nanofibers were
9 significantly increased. ERK pathway activation is also strongly related with the increase
10 of integrin β 1 expression, and we found that both proteins were highly expressed in cells
11 on L-VV nanofibers. Integrins interact with ECM fibrils and intracellular actin filaments
12 through cytosolic linker proteins, which facilitate the mechanical connection of
13 intracellular and extracellular environment of cells to raise specific cellular responses.⁴⁴
14 In addition, integrins act as transducers in the cellular sensing of physical forces that are
15 exerted through the surrounding environment of cells.^{44, 45} The L- form of cylindrical
16 nanofibers increased integrin β 1 expression and promoted the activation of ERK
17 pathway, which stimulated the osteogenic differentiation of MSCs. This activation was
18 also linked to the activation of F-actin, leading to the formation of well-organized stress
19 fibers that were observed by confocal microscopy through actin staining. Interestingly,
20 this effect of cylindrical nanofibers was much more advanced in the L-form compared to
21 the D-form, which underlines the importance of the chiral signature of supramolecular
22 nanofiber networks for the recognition by integrin receptors and the activation of
23 signaling pathways.

24 For the analysis of focal adhesion complexes, vinculin expression was evaluated by using
25 both confocal microscopy and flow cytometry (Figure S19B). Vinculin is responsible for
26 bearing the forces exerted on the focal adhesion complex, and its presence is essential for
27 anchoring the complex to the surrounding actin network.^{44, 46} According to confocal
28 microscopy analyses, culturing on L-VV nanofibers significantly increased focal
29 adhesion complex formation on the lamellipodial membranes of cells compared to other
30 groups. In addition, flow cytometry results revealed a marked increase in the expression
31 of vinculin in cells cultured on D-VV nanofibers, which may have enhanced the

1 formation of focal adhesion complexes at later days of culture; albeit not as quickly as L-
2 VV nanofibers. Interestingly, we also observed a similar increase in the expression of
3 Integrin $\alpha 2$, which forms a heterodimer complex with Integrin $\beta 1$ and has been shown to
4 bind to collagen and fibrinogen fibrils in ECM⁴⁵ (Figure S19A). This result further
5 supported the hypothesis that D-VV nanofibers increased the expression of fibrillar
6 protein-binding integrins, which can be later recruited for the formation of focal adhesion
7 complexes, but not as quickly as on L-VV nanofibers. In addition, an increase in FAK
8 protein expression was observed in most of the cells on D-VV nanofibers, at a level that
9 was significantly higher than other peptide nanofibers (Figure S19C). Overall, these
10 findings all indicate that culturing on D-VV nanofibers caused a delayed response in the
11 formation of focal adhesion complexes when compared to L-VV nanofibers. While the
12 spreading areas of L-VV and D-VV groups are similar, long-term adhesive forces have
13 been shown to influence cellular behavior to a greater extent than spreading, suggesting
14 that mechanotransduction may be more prominent on L-VV (as evidenced by higher
15 YAP nuclearization and osteogenic differentiation) despite comparable cell areas. In
16 addition, while vinculin and FAK expression were higher on D-VV nanofibers, vinculin
17 in this group was confined to the cytoplasm and the number of focal adhesions was less
18 compared to L-VV (Figure S19 and Figure 4). Consequently, we believe that D-VV
19 shows an effect that is similar to but less pronounced than L-VV, such that the cells are
20 able to spread but do not establish strong connections with the substrate. The higher
21 expressions of integrin alpha-2, FAK and vinculin may be a result of the fact that cells on
22 D-VV are at a stage where focal adhesion complexes are in the process of being
23 established, while the mature connections formed on L-VV are maintained by
24 comparatively lower expressions of focal adhesion point-related proteins⁶⁴. As a
25 functional demonstration of this effect, cells spread more on D-VV after 3 days of
26 incubation than 1 day incubation (Figure S16).

27 The twisted ribbon-like nanofibers limited the spreading of MSCs, decreased focal
28 adhesion points and actin fibers, and deactivated the ERK pathway through diminished
29 Integrin $\beta 1$ expression. Matrix-induced cell rounding has previously been reported to
30 correspond directly to decreases in focal adhesion and ERK pathway activation,⁵³ which
31 is caused by alterations in the mechanical feedback that occurs between collagen tethers

1 and anchoring proteins on the cell membrane. As such, the suppression of integrin
2 ligation in the twisted ribbon-like nanofibers appears to limit the adhesion of rMSCs
3 resulting in insufficient mechanical feedback and limited ERK/MAPK pathway signaling.
4 Although osteogenesis was not observed on FF containing nanofibers, chondrogenesis
5 was significantly promoted on these scaffolds (and especially on the D-FF form), which
6 is an interesting result that warrants further characterization and may be utilized for the
7 development of smart biomaterial platforms for cartilage tissue engineering.

8 The effect of mechanical cues from the extracellular environment was also reflected on
9 the gene expression profiles of rMSCs for the previously mentioned cellular signaling
10 pathways. We observed that the cytosolic/nuclear localization of YAP protein was altered
11 in cells grown on supramolecular peptide nanofibers. YAP/TAZ is a pair of
12 transcriptional co-activators that play critical roles in organ growth and have been shown
13 to be essential in the mechanotransduction of cells through their activity as nuclear
14 sensors of stiffness.^{47, 65} In particular, extracellular mechanical signals resulting from
15 substrate effects or excessive cell growth trigger the organization of F-actin and enhance
16 the nuclear localization of YAP.⁶⁵ Similarly, we found that MSCs on L-form cylindrical
17 nanofibers (L-VV) had stronger integrin $\beta 1$ expression, activated ERK signaling, and
18 nuclear YAP activity compared to other groups. In contrast, cells on twisted ribbon-like
19 nanofibers had low proliferation, confined cellular spreading and less focal adhesion,
20 which resulted in the cytoplasmic retention of YAP. Even though the D-form of
21 cylindrical nanofibers (D-VV) had a similar bulk morphology to the L-form, cells on the
22 L-form had higher nuclear YAP activity compared to the D-form, suggesting that
23 different chiral signals result in different routes of mechanotransduction in cells. The
24 correlation between the decrease in proliferation and YAP activity also agrees with recent
25 studies on the regulation of YAP/TAZ nuclear activity and proliferation by N-cadherin
26 and RGD-incorporated hydrogels.⁴⁸

27 **Conclusion**

28 In summary, we showed that mechanosensitive pathways are selectively activated in stem
29 cells depending on the morphology and chirality of the peptide nanofibers in the
30 microenvironment. The mechanosensation and transduction controlled the activity of the
31 YAP/TAZ complex, which relays the effects of the microenvironment to inside of the

cells through integrin β 1 receptor binding and subsequent cytosolic protein activations in the ERK pathway, ultimately result in the nuclear localization of YAP in response to L amino acid containing cylindrical nanofibers. However, this response was modulated not only by mechanical signaling, but also by the chemical signatures of the nanofibers and their chiral features. Morphology of the nanofibers was primarily responsible for initiating the cellular responses, but had synergistic effects with chiral signals. L-enantiomers found in natural proteins were observed to exhibit an increased effect on cell responses when introduced on cylindrical nanofibers; enhancing their capacity for promoting osteogenic differentiation even without incorporation of a bioactive epitope. D-enantiomers, on the other hand, had a pronounced effect on chondrogenesis when introduced in ribbon-like nanofibers. Overall, these results show that the morphology and chirality of the nanofibers can be exploited for selective differentiation of stem cells for regenerative medicine applications. In addition, it is interesting to note that fundamental differences in hydrogen bond formation between peptide enantiomers may have played an important role during the early evolution of proteins, potentially accounting for the fundamental chiral asymmetry that exists in all known life.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Chemical structures of PAs, primer sequences for qRT-PCR experiments, peptide nanofiber compositions, LC-MS results, TEM images of PA nanofibers, SAXS analyses, molecular dynamics simulations, rheological characterizations of nanofibers, AFM characterizations, Alamar Blue, Live/Dead and Adhesion assay results for rMSCs, additional spreading results for HUVECs and hFibs, chondrogenic differentiation analyses of rMSCs, protein expression analyses of Integrin α 2 and FAK by western blotting, protein expression analyses of Vinculin by flow cytometry analyses, confocal microscopy images of actin filaments of rMSCs.

AUTHOR INFORMATION

Corresponding Author

**E-mail: atekinay@bilkent.edu.tr (A.B.T.)*

**E-mail: mguler@uchicago.edu (M.O.G.)*

Author Contributions

In vitro cell culture studies were designed and performed by E.A., O.U., A.B.T.; chemical synthesis and characterizations were designed and performed by M.H.K., E.A., R.G., M.O.G.; AFM measurements and analyses were performed by B.D., A.E.T., E.A., A.D.O and A.D.; molecular dynamics simulations were performed by S.B.; SAXS analysis were performed by D.H.M., V.C., C.E.G., and I.H.. The manuscript was written by E.A, A.B.T., and M.O.G. with contributions of all authors. All authors have given approval to the final version of the manuscript.

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NOTES

The authors declare no competing financial interest.

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ABBREVIATIONS

PA, peptide amphiphile; ECM, extracellular matrix; FBS, fetal bovine serum; CD, circular dichroism; TEM, transmission electron microscopy; SEM, scanning electron microscopy; qRT-PCR, quantitative real time polymerase chain reaction; prep-HPLC, preparative high performance liquid chromatography; s.e.m, standard error of mean.

REFERENCES

1. Guilak, F.; Cohen, D. M.; Estes, B. T.; Gimble, J. M.; Liedtke, W.; Chen, C. S., Control of stem cell fate by physical interactions with the extracellular matrix. *Cell stem cell* **2009**, 5 (1), 17-26.
2. Koide, T., Designed triple-helical peptides as tools for collagen biochemistry and matrix engineering. *Philos. Trans. R. Soc., B* **2007**, 362 (1484), 1281-1291.
3. Knight, C. G.; Morton, L. F.; Peachey, A. R.; Tuckwell, D. S.; Farndale, R. W.; Barnes, M. J., The Collagen-binding A-domains of Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ Recognize the Same Specific Amino Acid Sequence, GFOGER, in Native (Triple-helical) Collagens. *J. Biol. Chem.* **2000**, 275 (1), 35-40.
4. Li, B.; Moshfegh, C.; Lin, Z.; Albuschies, J.; Vogel, V., Mesenchymal stem cells exploit extracellular matrix as mechanotransducer. *Sci. Rep.* **2013**, 3.
5. Muiznieks, L. D.; Keeley, F. W., Molecular assembly and mechanical properties of the extracellular matrix: A fibrous protein perspective. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **2013**, 1832 (7), 866-875.
6. Das, R. K.; Zouani, O. F.; Labrugère, C.; Oda, R.; Durrieu, M.-C., Influence of Nanohelical Shape and Periodicity on Stem Cell Fate. *ACS Nano* **2013**, 7 (4), 3351-3361.
7. Buxboim, A.; Ivanovska, I. L.; Discher, D. E., Matrix elasticity, cytoskeletal forces and physics of the nucleus: how deeply do cells 'feel' outside and in? *J. Cell Sci.* **2010**, 123 (3), 297-308.
8. Liu, G. F.; Zhang, D.; Feng, C. L., Control of Three- Dimensional Cell Adhesion by the Chirality of Nanofibers in Hydrogels. *Angew. Chem., Int. Ed.* **2014**, 53 (30), 7789-7793.
9. Sun, T.; Han, D.; Rhemann, K.; Chi, L.; Fuchs, H., Stereospecific interaction between immune cells and chiral surfaces. *J. Am. Chem. Soc.* **2007**, 129 (6), 1496-1497.
10. Zhang, M.; Qing, G.; Sun, T., Chiral biointerface materials. *Chem. Soc. Rev.* **2012**, 41 (5), 1972-1984.
11. Jakschitz, T. A.; Rode, B. M., Chemical evolution from simple inorganic compounds to chiral peptides. *Chem. Soc. Rev.* **2012**, 41 (16), 5484-5489.
12. Krause, E.; Bienert, M.; Schmieder, P.; Wenschuh, H., The helix-destabilizing propensity scale of D-amino acids: the influence of side chain steric effects. *J. Am. Chem. Soc.* **2000**, 122 (20), 4865-4870.

- 1 13. Arslan, E.; Garip, I. C.; Gulseren, G.; Tekinay, A. B.; Guler, M. O., Bioactive
2 Supramolecular Peptide Nanofibers for Regenerative Medicine. *Adv. Healthcare Mater.*
3 **2014**, 3 (9), 1357-1376.
- 4 14. Barker, T. H., The role of ECM proteins and protein fragments in guiding cell
5 behavior in regenerative medicine. *Biomaterials* **2011**, 32 (18), 4211-4214.
- 6 15. Webber, M. J.; Kessler, J.; Stupp, S. I., Emerging peptide nanomedicine to
7 regenerate tissues and organs. *J. Intern. Med.* **2010**, 267 (1), 71-88.
- 8 16. Tansik, G.; Kilic, E.; Beter, M.; Demiralp, B.; Sendur, G. K.; Can, N.; Ozkan, H.;
9 Ergul, E.; Guler, M. O.; Tekinay, A. B., A glycosaminoglycan mimetic peptide nanofiber
10 gel as an osteoinductive scaffold. *Biomater. Sci.* **2016**, 4 (9), 1328-1339.
- 11 17. Ceylan, H.; Kocabey, S.; Tekinay, A. B.; Guler, M. O., Surface-adhesive and
12 osteogenic self-assembled peptide nanofibers for bioinspired functionalization of titanium
13 surfaces. *Soft Matter* **2012**, 8 (14), 3929-3937.
- 14 18. Gulseren, G.; Yasa, I. C.; Ustahuseyin, O.; Tekin, E. D.; Tekinay, A. B.; Guler,
15 M. O., Alkaline phosphatase-mimicking peptide nanofibers for osteogenic differentiation.
16 *Biomacromolecules* **2015**, 16 (7), 2198-2208.
- 17 19. Senturk, B.; Cubuk, M. O.; Ozmen, M. C.; Aydin, B.; Guler, M. O.; Tekinay, A.
18 B., Inhibition of VEGF mediated corneal neovascularization by anti-angiogenic peptide
19 nanofibers. *Biomaterials* **2016**, 107, 124-132.
- 20 20. Yasa, I. C.; Gunduz, N.; Kilinc, M.; Guler, M. O.; Tekinay, A. B., Basal Lamina
21 Mimetic Nanofibrous Peptide Networks for Skeletal Myogenesis. *Sci. Rep.* **2015**, 5,
22 16460-16460.
- 23 21. Markvoort, A. J.; Ten Eikelder, H. M.; Hilbers, P. A.; de Greef, T. F.; Meijer, E.,
24 Theoretical models of nonlinear effects in two-component cooperative supramolecular
25 copolymerizations. *Nat. Commun.* **2011**, 2, 509.
- 26 22. Zhang, L.; Wang, X.; Wang, T.; Liu, M., Supramolecular Gels: Tuning Soft
27 Nanostructures in Self- assembled Supramolecular Gels: From Morphology Control to
28 Morphology- Dependent Functions (Small 9- 10/2015). *Small* **2015**, 11 (9-10), 1024-
29 1024.
- 30 23. Chen, G.; Ushida, T.; Tateishi, T., Development of biodegradable porous
31 scaffolds for tissue engineering. *Mater. Sci. Eng., C* **2001**, 17 (1), 63-69.

- 1 24. Green, D. W.; Lee, J.-M.; Kim, E.-J.; Lee, D.-J.; Jung, H.-S., Chiral Biomaterials:
2 From Molecular Design to Regenerative Medicine. *Adv. Mater. Interfaces* **2016**, 3 (6),
3 1500411-n/a.
- 4 25. Breßler, I.; Kohlbrecher, J.; Thünemann, A. F., SASfit: a tool for small-angle
5 scattering data analysis using a library of analytical expressions. *J. Appl. Crystallogr.*
6 **2015**, 48 (5), 1587-1598.
- 7 26. Castelletto, V.; Cheng, G.; Stain, C.; Connon, C.; Hamley, I., Self-assembly of a
8 peptide amphiphile containing L-carnosine and its mixtures with a multilamellar vesicle
9 forming lipid. *Langmuir* **2012**, 28 (31), 11599-11608.
- 10 27. Castelletto, V.; Gouveia, R. M.; Connon, C. J.; Hamley, I. W., New RGD-peptide
11 amphiphile mixtures containing a negatively charged diluent. *Faraday Discuss.* **2013**,
12 166, 381-397.
- 13 28. Pabst, G.; Rappolt, M.; Amenitsch, H.; Laggner, P., Structural information from
14 multilamellar liposomes at full hydration: full q-range fitting with high quality x-ray data.
15 *Phys. Rev. E* **2000**, 62 (3), 4000.
- 16 29. Tekin, E. D., Molecular dynamics simulations of self-assembled peptide
17 amphiphile based cylindrical nanofibers. *RSC Adv.* **2015**, 5 (82), 66582-66590.
- 18 30. Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.;
19 Chipot, C.; Skeel, R. D.; Kale, L.; Schulten, K., Scalable molecular dynamics with
20 NAMD. *J. Comput. Chem.* **2005**, 26 (16), 1781-1802.
- 21 31. MacKerell Jr, A. D.; Bashford, D.; Bellott, M.; Dunbrack Jr, R. L.; Evanseck, J.
22 D.; Field, M. J.; Fischer, S.; Gao, J.; Guo, H.; Ha, S., All-atom empirical potential for
23 molecular modeling and dynamics studies of proteins. *J. Chem. Phys. b* **1998**, 102 (18),
24 3586-3616.
- 25 32. Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T.; Lee, H.; Pedersen, L. G.,
26 A smooth particle mesh Ewald method. *J. Chem. Phys.* **1995**, 103 (19), 8577-8593.
- 27 33. Roe, D. R.; Cheatham III, T. E., PTRAJ and CPPTRAJ: software for processing
28 and analysis of molecular dynamics trajectory data. *J. Chem. Theory Comput.* **2013**, 9
29 (7), 3084-3095.
- 30 34. Humphrey, W.; Dalke, A.; Schulten, K., VMD: visual molecular dynamics. *J.*
31 *Mol. Graphics* **1996**, 14 (1), 33-38.

- 1 35. Niece, K. L.; Hartgerink, J. D.; Donners, J. J.; Stupp, S. I., Self-assembly
2 combining two bioactive peptide-amphiphile molecules into nanofibers by electrostatic
3 attraction. *J. Am. Chem. Soc.* **2003**, *125* (24), 7146-7147.
- 4 36. Pashuck, E. T.; Cui, H.; Stupp, S. I., Tuning supramolecular rigidity of peptide
5 fibers through molecular structure. *J. Am. Chem. Soc.* **2010**, *132* (17), 6041-6046.
- 6 37. Arslan, E.; Guler, M. O.; Tekinay, A. B., Glycosaminoglycan-mimetic signals
7 direct the osteo/chondrogenic differentiation of mesenchymal stem cells in a three-
8 dimensional peptide nanofiber extracellular matrix mimetic environment.
9 *Biomacromolecules* **2016**, *17* (4), 1280-1291.
- 10 38. Pashuck, E. T.; Stupp, S. I., Direct observation of morphological transformation
11 from twisted ribbons into helical ribbons. *J. Am. Chem. Soc.* **2010**, *132* (26), 8819-8821.
- 12 39. Garifullin, R.; Guler, M. O., Supramolecular chirality in self-assembled peptide
13 amphiphile nanostructures. *Chem. Commun.* **2015**, *51* (62), 12470-12473.
- 14 40. Micsonai, A.; Wien, F.; Kernya, L.; Lee, Y.-H.; Goto, Y.; Réfrégiers, M.; Kardos,
15 J., Accurate secondary structure prediction and fold recognition for circular dichroism
16 spectroscopy. *Proc. Natl. Acad. Sci.* **2015**, *112* (24), E3095-E3103.
- 17 41. Adler, A. J.; Greenfield, N. J.; Fasman, G. D., [27] Circular dichroism and optical
18 rotatory dispersion of proteins and polypeptides. *Methods Enzymol.* **1973**, *27*, 675-735.
- 19 42. Hovmöller, S.; Zhou, T.; Ohlson, T., Conformations of amino acids in proteins.
20 *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2002**, *58* (5), 768-776.
- 21 43. Park, Y. B.; Kim, Y. Y.; Oh, S. K.; Chung, S. G.; Ku, S.-Y.; Kim, S. H.; Choi, Y.
22 M.; Moon, S. Y., Alterations of proliferative and differentiation potentials of human
23 embryonic stem cells during long-term culture. *Exp. Mol. Med.* **2008**, *40* (1), 98-108.
- 24 44. Schwartz, M. A., Integrins and extracellular matrix in mechanotransduction. *Cold
25 Spring Harbor Perspect. Biol.* **2010**, *2* (12), a005066.
- 26 45. Prowse, A. B. J.; Chong, F.; Gray, P. P.; Munro, T. P., Stem cell integrins:
27 Implications for ex-vivo culture and cellular therapies. *Stem Cell Res.* **2011**, *6* (1), 1-12.
- 28 46. Chanet, S.; Martin, A. C., Mechanical force sensing in tissues. *Prog. Mol. Biol.
29 Transl. Sci.* **2014**, *126*, 317.

- 1 47. Aragona, M.; Panciera, T.; Manfrin, A.; Giulitti, S.; Michielin, F.; Elvassore, N.;
2 Dupont, S.; Piccolo, S., A mechanical checkpoint controls multicellular growth through
3 YAP/TAZ regulation by actin-processing factors. *Cell* **2013**, *154* (5), 1047-1059.
- 4 48. Cosgrove, B. D.; Mui, K. L.; Driscoll, T. P.; Caliri, S. R.; Mehta, K. D.; Assoian,
5 R. K.; Burdick, J. A.; Mauck, R. L., N-cadherin adhesive interactions modulate matrix
6 mechanosensing and fate commitment of mesenchymal stem cells. *Nat. Mater.* **2016**,
7 *advance online publication*.
- 8 49. Luo, Z.; Zhang, S., Designer nanomaterials using chiral self-assembling peptide
9 systems and their emerging benefit for society. *Chem. Soc. Rev.* **2012**, *41* (13), 4736-
10 4754.
- 11 50. Aggeli, A.; Nyrkova, I. A.; Bell, M.; Harding, R.; Carrick, L.; McLeish, T. C.;
12 Semenov, A. N.; Boden, N., Hierarchical self-assembly of chiral rod-like molecules as a
13 model for peptide β -sheet tapes, ribbons, fibrils, and fibers. *Proc. Natl. Acad. Sci.* **2001**,
14 *98* (21), 11857-11862.
- 15 51. Gibaud, T.; Barry, E.; Zakhary, M. J.; Henglin, M.; Ward, A.; Yang, Y.; Berciu,
16 C.; Oldenbourg, R.; Hagan, M. F.; Nicastro, D., Reconfigurable self-assembly through
17 chiral control of interfacial tension. *Nature* **2012**, *481* (7381), 348-351.
- 18 52. Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E., Matrix elasticity directs
19 stem cell lineage specification. *Cell* **2006**, *126* (4), 677-689.
- 20 53. Trappmann, B.; Gautrot, J. E.; Connelly, J. T.; Strange, D. G.; Li, Y.; Oyen, M.
21 L.; Stuart, M. A. C.; Boehm, H.; Li, B.; Vogel, V., Extracellular-matrix tethering
22 regulates stem-cell fate. *Nat. Mater.* **2012**, *11* (7), 642-649.
- 23 54. Park, J. S.; Chu, J. S.; Tsou, A. D.; Diop, R.; Tang, Z.; Wang, A.; Li, S., The
24 effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to
25 TGF- β . *Biomaterials* **2011**, *32* (16), 3921-3930.
- 26 55. Evans, N. D.; Minelli, C.; Gentleman, E.; LaPointe, V.; Patankar, S. N.;
27 Kallivretaki, M.; Chen, X.; Roberts, C. J.; Stevens, M. M., Substrate stiffness affects
28 early differentiation events in embryonic stem cells. *Eur. cell mater.* **2009**, *18* (1), e13.
- 29 56. Mondal, S.; Varenik, M.; Bloch, D. N.; Atsmon-Raz, Y.; Jacoby, G.; Adler-
30 Abramovich, L.; Shimon, L. J.; Beck, R.; Miller, Y.; Regev, O., A minimal length rigid

- 1 helical peptide motif allows rational design of modular surfactants. *Nat. Commun.* **2017**,
2 8.
- 3 57. Eyckmans, J.; Lin, G. L.; Chen, C. S., Adhesive and mechanical regulation of
4 mesenchymal stem cell differentiation in human bone marrow and periosteum-derived
5 progenitor cells. *Biol. Open* **2012**, BIO20122162.
- 6 58. Kilian, K. A.; Bugarija, B.; Lahn, B. T.; Mrksich, M., Geometric cues for
7 directing the differentiation of mesenchymal stem cells. *Proc. Natl. Acad. Sci.* **2010**, 107
8 (11), 4872-4877.
- 9 59. Akiyama, H.; Kim, J.-E.; Nakashima, K.; Balmes, G.; Iwai, N.; Deng, J. M.;
10 Zhang, Z.; Martin, J. F.; Behringer, R. R.; Nakamura, T., Osteo-chondroprogenitor cells
11 are derived from Sox9 expressing precursors. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, 102
12 (41), 14665-14670.
- 13 60. Park, H.-J.; Zhang, Y.; Georgescu, S. P.; Johnson, K. L.; Kong, D.; Galper, J. B.,
14 Human umbilical vein endothelial cells and human dermal microvascular endothelial
15 cells offer new insights into the relationship between lipid metabolism and angiogenesis.
16 *Stem Cell Rev. Rep.* **2006**, 2 (2), 93-101.
- 17 61. Gao, L.; McBeath, R.; Chen, C. S., Stem Cell Shape Regulates a Chondrogenic
18 versus Myogenic Fate through Rac1 and N-cadherin. *Stem cells (Dayton, Ohio)* **2010**, 28
19 (3), 564-572.
- 20 62. Jaiswal, R. K.; Jaiswal, N.; Bruder, S. P.; Mbalaviele, G.; Marshak, D. R.;
21 Pittenger, M. F., Adult Human Mesenchymal Stem Cell Differentiation to the Osteogenic
22 or Adipogenic Lineage Is Regulated by Mitogen-activated Protein Kinase. *J. Biol. Chem.*
23 **2000**, 275 (13), 9645-9652.
- 24 63. Li, C.-S.; Zheng, Z.; Su, X.-X.; Wang, F.; Ling, M.; Zou, M.; Zhou, H.,
25 Activation of the Extracellular Signal-Regulated Kinase Signaling Is Critical for Human
26 Umbilical Cord Mesenchymal Stem Cell Osteogenic Differentiation. *BioMed Res. Int.*
27 **2016**, 2016, 10.
- 28 64. Hanein, D.; Horwitz, A. R., The structure of cell–matrix adhesions: the new
29 frontier. *Curr. Opin. Cell Biol.* **2012**, 24 (1), 134-140.

65. Chen, W.; Shao, Y.; Li, X.; Zhao, G.; Fu, J., Nanotopographical surfaces for stem cell fate control: Engineering mechanobiology from the bottom. *Nano Today* **2014**, *9* (6), 759-784.
66. Mammadov, R.; Mammadov, B.; Toksoz, S.; Aydin, B.; Yagci, R.; Tekinay, A. B.; Guler, M. O., Heparin Mimetic Peptide Nanofibers Promote Angiogenesis. *Biomacromolecules* **2011**, *12* (10), 3508-3519.
67. Mammadov, R.; Mammadov, B.; Guler, M. O.; Tekinay, A. B., Growth Factor Binding on Heparin Mimetic Peptide Nanofibers. *Biomacromolecules* **2012**, *13* (10), 3311-3319.
68. Lim, J. Y.; Donahue, H. J., Cell sensing and response to micro- and nanostructured surfaces produced by chemical and topographic patterning. *Tissue eng.* **2007**, *13* (8), 1879-1891.

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